An Alternative Approach for a Genetic Vaccine: HSV-1 Amplicon Vector Encoded Heterologous Virus-Like Particles

Dissertation

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Table of contents

Summary

The overall goal of this thesis was to evaluate if herpes simplex virus type-1 (HSV-1) amplicon vectors are possible candidate vectors to establish a new vaccine vector platform against rotavirus (RV) infections and Foot-and-mouth disease virus (FMDV).

RV infections lead to severe gastroenteritis in young children, killing more than half a million infants worldwide per year. Almost all children both in developing and developed countries are infected with RV during their first years of life and even advanced levels of sanitation and hygiene appear unable to control the spread of RV infections, which highlights the urgent need for the development of an effective prophylactic anti-RV vaccine.

FMDV is one of the most devastating viruses affecting cloven-hoofed animals, like cattle, sheep, goats and pigs. Foot-and mouth disease (FMD) represents one of the most important epidemics of farm animals and continues to be of major economic importance across the world. FMDV spreads rapidly in a susceptible population and presently, the disease is controlled by slaughter of infected or in-contact animals, and quarantine and decontamination for the whole region. An effective and safe vaccine against FMD would be most desirable.

Virus-like particles (VLPs) are most promising candidates for the development of vaccines, as they are very similar to the equivalent virus, but safer than attenuated virus vaccines, since they do not contain genetic material. VLPs mimic the overall structure of virus particles and are therefore readily recognized by the immune system as they represent viral antigens in a more authentic conformation.

In the present work, HSV-1 amplicon vectors have been developed that co-express the structural genes required for capsid assembly, of the two RNA viruses, FMDV and RV. The designed amplicon vectors provide a high safety level as they can be produced by using a helper virus-free packaging system leading to the absence of expression of any HSV-1 gene. The high transgene capacity (150 kbp) allows the insertion of several genes, and the simultaneous expression of the different structural genes of FMDV and RV was achieved by introducing internal ribosome entry site (IRES) sequences. Expression of the individual genes alone or in combination was demonstrated by Western and immune fluorescence analyses. The capability of the vectors to support the production of RV and FMDV VLPs in the vector-transduced cells was demonstrated by electron microscopy.

Inoculation of mice with these vectors resulted in the expression of viral antigens, followed by induction of immune responses and a variable level of protection against challenge with a high dose of wild type virus. Moreover, the results obtained by vaccinating mice with inactivated vectors showed that induction of immune responses required de novo synthesis of proteins from the vectors and the contamination of vector stocks with preformed proteins is

not contributing to the immune responses, as demonstrated with inactivated amplicon vectors that do not support protein synthesis. In the case of FMDV amplicon vectors, induction of immune responses and protection against challenge were higher with amplicon vectors than with adenovirus vectors expressing the same FMDV proteins and required no adjuvants.

Taken together, the results presented in this work suggest that HSV-1 amplicon vectors are attractive candidates for the development of complex but safe genetic vaccines against viral infections.

Zusammenfassung

Rotaviren zählen bei Mensch und Tier weltweit zu den wichtigsten Erregern schwerer, dehydrierender Gastroenteritis. Weltweit führen Rotavirusinfektionen jährlich zu ca. 600'000 Todesfällen bei Kleinkindern unter 5 Jahren, und 30-80% aller Hospitalisierungen sind auf Rotavirusinfektionen zurückzuführen. Vor allem in Entwicklungsländern und Dritte Welt Ländern beträgt die Letalität bis zu 98%, da das Gesundheitssystem schlecht ausgebaut ist.
Rotaviren sind als ein individueller Genus (Gattung) in der Familie der *Reoviridae* klassifiziert. Ihr Durchmesser beträgt 70-75 nm, sie sind icosahedral, dreischichtig und unbehüllt. Das virale Genom wird durch das sogenannte Core, bestehend aus 120 Kopien von VP2, umschlossen. Die VP2 Schicht ist durch die mittlere Schicht umschlossen, die aus 780 Kopien des Hauptproteins VP6 besteht. Die äußerste Schicht der Hülle wird aus zwei Proteinen gebildet, dem Glykoprotein VP7 und dem Spikeprotein VP4. Das Virion ist komplex und erscheint im Elektronenmikroskop als Rad-ähnliche Struktur, deshalb der Name Rotavirus (Latein rota = Rad). Das Genom besteht aus 11 Segmenten doppelsträngiger RNA, die zusammen für 6 Strukturproteine (VP1-VP7) und, abhängig vom Virusstamm, 5 oder 6 Nichtstrukturproteine (NSP1-NSP6) kodieren. Das segmentierte Genom erlaubt die Bildung von Reassortanten (Genaustausch zwischen unterschiedlichen Rotaviren) und ermöglicht damit die Entstehung neuer Rotavirus Stämme und eine grosse Diversität dieser Viren.
Maul und Klauen Seuche (MKS) ist eine akute, hochkontagiöse Viruserkrankung der Wiederkäuer und der Schweine. MKS ist weltweit eine der verheerendsten Viruserkrankungen landwirtschaftlicher Nutztiere, da die Seuche innerhalb kürzester Zeit alle Klauentiere eines Betriebes befallen kann und schwere wirtschaftliche Schäden verursacht.
Das MKS Virus gehört zur Familie *Picornaviridae* (pico = klein), Gattung *Aphtovirus* und ist mit einem Durchmesser von 25-30 nm eines der kleinsten RNA Viren. Das Virion ist unbehüllt und hat eine icosahedrale Symmetrie. Die Proteinhülle (Kapsid) besteht aus einem dicht gepackten Arrangement von 60 Einheiten (Protomeren), wobei jedes aus 4 Strukturproteinen besteht, VP1, VP2, VP3 und VP4, wobei VP4 an der Innenseite des Kapsids lokalisiert ist und mit dem RNA Genom assoziiert ist. Das Genom besteht aus einzelsträngiger RNA mit positiver Polarität, ist nicht segmentiert und enthält einen einzigen offenen Leserahmen (ORF), der für ein Vorläuferprotein (Polyprotein) kodiert, welches noch während seiner Synthese proteolytisch in die verschiedenen Viruskomponenten gespalten wird, die Struktur- und Nichtstrukturproteine, sowie die viralen Enzyme. Die P1 Region des Vorläuferproteins enthält die Strukturproteine VP1-4; die P2 und P3 Regionen die Nichtstruktur-proteine, welche eine Rolle bei der Replikation spielen. Die Spaltung des Polyproteins erfolgt durch Virus-eigene Proteasen.

Das Ziel dieser Arbeit war, mit Hilfe von sogennanten Herpes simplex Virus Typ 1 (HSV-1) Amplikon Vektoren die Strukturgene von Rotaviren und MKS Viren in Zellen zu bringen, wo die Proteine der jeweiligen Virushülle hergestellt werden, die sich dann zu sogenannten virus-ähnlichen Partikeln (VLPs, virus-like particles) zusammenfügen, also leere Virushüllen, die aussehen wie das Virus, aber kein Genom besitzen. Die Strukturgene wurden in Amplikonplasmide kloniert, welche durch die Ko-Transfektion von Zellen mit dem Amplikonplasmid und einem HSV-1 Helfergenom in infektiöse Amplikonpartikel verpackt wurden, welche dann zur Transduktion von Zellen benützt wurden.

Im Fall des Rotavirus exprimieren die Amplikonvektoren die drei Strukturproteine VP2, VP6 und VP7, welche zusammen das dreischichtige Rotaviruskapsid bilden. Für das MKS Virus wurde das Vorläuferprotein P1, welches die vier Strukturproteine der Virushülle VP1, VP2, VP3 und VP4 enthält zusammen mit der Virus-eigenen Protease 3C, welches das Vorläuferprotein spaltet, in die Vektoren kloniert.

Mittels Immunfluoresezenz und Western Blot konnte die Synthese der Proteine in Vektor-infizierten Zellen mittels jeweiligen Protein-spezifischen Antikörpern nachgewiesen werden, ebenso konnte mit Elektronenmikroskopie gezeigt werden, dass sich die Strukturproteine tatsächlich zu VLPs zusammenfügen.

Um zu untersuchen, ob sich die Amplikon Vektoren als eine mögliche Alternative für die Entwicklung von neuen Impfstoffen eignen, wurden Mäuse mit den Vektoren geimpft und die spezifische Antikörperantwort untersucht. Es konnte gezeigt werden, dass die Amplikonvektoren eine Virus-spezifische Immunreaktion auslösen und dass diese Antwort auf der Neusynthese der Proteine vom Vektor beruht, da fast keine Antikörper gebildet wurden, wenn die Vektoren vorher inaktiviert wurden.

Desweiteren wurde getestet, ob die Mäuse gegen eine Infektion geschützt sind, wozu ein sogenanntes Challenge Experiment durchgeführt wurde, wobei die vorher geimpften Mäuse mit Virus infiziert wurden. In beiden Fällen konnte gezeigt werden, dass die geimpften Mäuse teilweise gegen die jeweilige Infektion geschützt waren.

Zusammenfassend kann aus den Resultaten dieser Arbeit geschlossen werden, dass die Amplikonvektoren ein gutes Instrument darstellen, um eine neue Art von Impfstoffen gegen MKS Virus und Rotavirus Infektionen herzustellen.

1 Introduction

1.1 Rotavirus

1.1.1 History

The first rotaviruses (RVs) described, based on pathology and epidemiology, were murine strains classified under the general description as the agents responsible for “epizootic diarrhea of infant mice” (EDIM) (12, 87). In 1963, the first RVs were visualized by electron microscopy (1) and in the same year also observed in rectal swabs of monkeys (66). The agents were described as 70 nm particles that had a wheel-like appearance when they are observed by negative-stain electron microscopy (Figure 1), a characteristic that suggested the name of the virus, “rota” viruses from the Latin word for wheel (34). The presence of such particles also in stool of calves with diarrhea demonstrated the association of this virus with a diarrheal disease in cattle (77). The correlation of the described virus with severe diarrhea in young children was reported first in 1973 by Bishop et al (9), when virus particles were found by electron microscopy in duodenal biopsies from children with acute gastroenteritis.

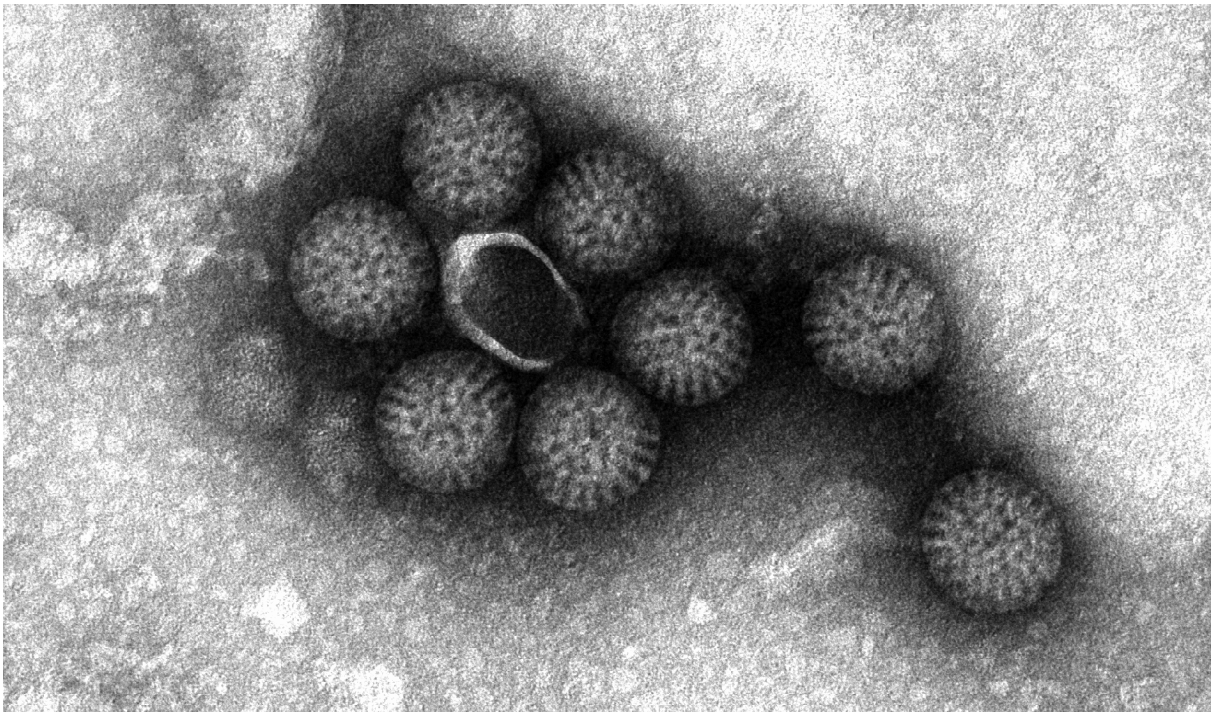

Figure 1: Rotavirus particle. Negative-stain electron microscopy of purified and inactivated Rotavirus strain SA11. (A. Laimbacher and E. Schraner, University of Zurich, Switzerland)

Besides their distinctive morphologic features, RVs were shown to share a group antigen (50) and were therefore classified as members of the genus Rotavirus within the family of the *Reoviridae* (70). The finding of particles in pigs with the same morphology but lacking the common group antigen (11, 101) led to the establishment of six additional RV groups (B to G) with the original RV strain classified as group A.

In a relatively short period, investigators from many countries reported the detection of rotaviruses in faeces of paediatric patients with diarrhoeal illness, and it was soon established that rotaviruses were the long–sought-after major viral etiologic agents of severe diarrhoea of infants and young children in both developed and developing countries, consistently outranking in importance other known etiologic agents of severe diarrhoea.

1.1.2 Virus Classification

The rotaviruses compose a genus within the family *Reoviridae* and share common morphologic and biochemical properties: (a) mature virus particles are about 75-100 nm in diameter and posses a triple–layered icosahedral protein capsid composed of an outer layer, intermediate layer and an inner core layer; (b) 60 spikes extend from the smooth surface of the outer shell; (c) particles contain an RNA-dependent RNA polymerase and other proteins capable to produce capped RNA transcripts; (d) the virus genome contains 11 segments of double-stranded RNA (dsRNA); (e) RVs of the same group are capable of genetic reassortment; (f) virus replication occurs in the cytoplasm of infected cells; (g) virus cultivation *in vitro* is facilitated by treatment with proteolytic enzymes, which enhances infectivity by cleavage of an outer capsid spike polypeptide; and (h) the viruses exhibit a unique morphogenic pathway (i.e., virus particles are formed by budding into ER) and enveloped particles are evident transiently at this stage of morphogenesis. Mature particles are non-enveloped, and these virions are liberated from infected cells by cell lysis or by non-classical vesicular transport in polarized epithelial cells (29, 31).

The classification scheme of rotaviruses is based on genome composition and immunological reactivities of three of its components: (i) The middle layer capsid protein VP6 designates the group, of which at least seven (A-G) are reported until now. Among group A RVs, two major subgroups (I and II) exist. (ii) A binary classification system based on the two surface proteins VP4 and VP7 has been established, by which G types (VP7-specific, G for glycoprotein) and P types (VP4-specific, P for protease-sensitive protein) are distinguished. So far, at least 16 G types and 27 P types have been detected, of which at least 11 G and 11 P types infect humans. Group A RVs are the most common cause of RV diseases in humans.

Various combinations of VP4 and VP7 types have been observed in RV isolates as VP4 and VP7 are encoded by different RNA segments, which leads to reassortments in double-infected cells.

1.1.3 Virion structure and proteins

The mature infectious rotavirus particle is made of three concentric icosahedral protein layers. The complete virion is called a triple-layered particle containing a non-enveloped, icosahedral capsid with a diameter of approximately 75-100 nm (100 nm including the

spikes). These three protein layers encapsidate the viral genome of 11 segments of ds RNA (Figure 2).

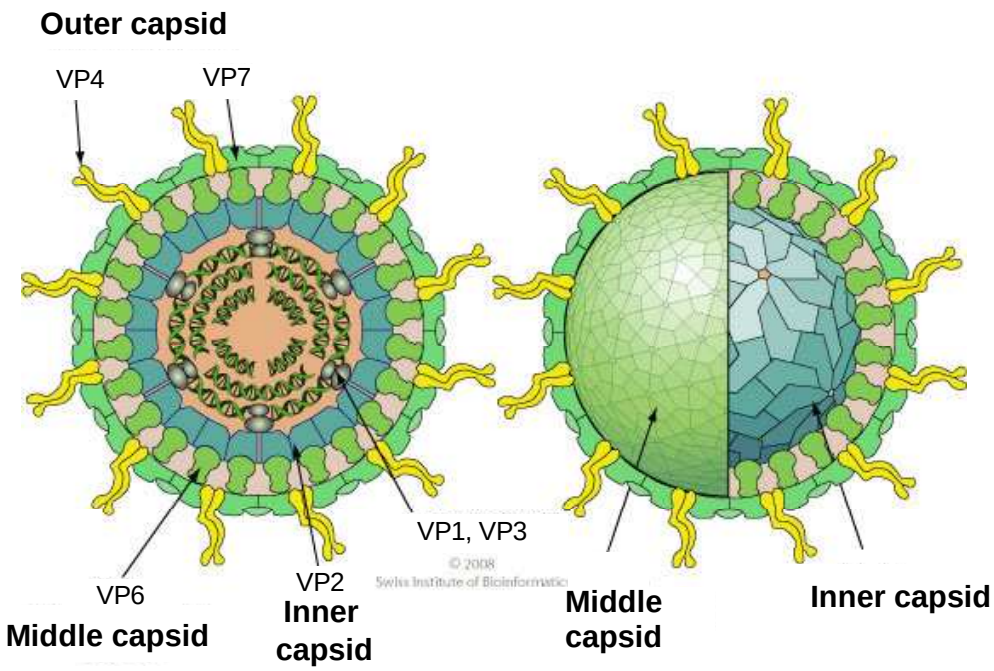

Figure 2: Virion structure of Rotavirus. The virion is composed of three protein layers mainly formed by four structural proteins: The inner capsid is formed by 120 copies of VP2 arranged as dimers; the middle capsid is made of 780 copies of VP6 forming trimers; the outermost layer is composed of 780 copies of VP7-trimers decorated with 60 spikes formed by 120 copies of VP4 (Adapted from ViralZone: www.expasy.org/viralzone, Swiss Institute of Bioinformatics).

The 11 ds RNA segments of the rotavirus genome encode for six structural and six non-structural proteins. Based on their molecular weights the structural proteins are named as viral protein (VP), with VP1 for the largest until VP8*, one of the two proteolytic fragments of VP4, as the smallest (Table 1). The capsid of the mature virion is formed by six structural proteins, whereas the six non-structural proteins (NSPs) are essential for virus replication cycle or interact with host proteins to influence pathogenesis or the immune response. They are found in infected cells but are not incorporated into the mature virion.

Table 1: dsRNA segments and proteins of rotavirus strain SA11

Genome Segment	Gene Product(s)	Protein Size kDa	Location in Virus Particle	Copies/ Particle	Functions and Properties
1	VP1	125	Inner capsid	12	RNA-dependent RNA polymerase, part of minimal replication complex, part of virion transcription complex with VP3, ss-RNA binding
2	VP2	102.4	Inner capsid	120	Inner capsid structural protein, non-specific ss & dsRNA-binding

					activity, required for replicase activity of VP1
3	VP3	98.1	Inner capsid	12	Guanylyltransferase, methyltransferase, non-specific ss & dsRNA binding, part of virion transcription complex with VP1
4	VP4	86.8	Outer capsid spike	120	VP4 dimers form spikes, hemagglutinin, cell attachment, P-type neutralizing antigen, cleavage by trypsin into VP5* and VP8* enhances infectivity
	VP5*	60			
	VP8*	28			
5	NSP1	58.7	Nonstructural	0	Associates with cytoskeleton, extensive sequence diversity between strains, role in suppressing host INFa response
6	VP6	44.8	Middle capsid	780	Major virion protein, homotrimeric structure forming middle layer, subgroup antigen, required for transcription
7	NSP3	34.6	Nonstructural	0	Homodimer, involved in translational regulation and host shut-off, RNA-binding
8	NSP2	36.7	Nonstructural	0	NTPase and helicase, RNA and NSP5 binding, involved in viroplasm formation
9	VP7	37.4	Outer capsid	780	Outer capsid structural glycoprotein, G-type neutralization antigen, rER transmembrane protein, Ca-binding
10	NSP4	20.2	Nonstructural	0	Viral enterotoxin, receptor for budding of double-layered particles through ER, rER transmembrane protein, modulates intracellular Ca levels and RNA replication, secreted cleavage product
11	NSP5	21.7	Nonstructural	0	Interacts with NSP2 and NSP6, homomultimers, hyperphosphorylated, component of viroplasm, essential for viral replication, protein kinase
	NSP6	11	Nonstructural	0	Product of 2nd out-of-frame ORF, interacts with NSP5, located in viroplasm

References: (4, 31); Mertens and Bamford, 2003, http://www.reoviridae.org/dsRNA_virus_proteins/Rotavirus.htm

One aim of this thesis was to produce virus-like particles (VLPs) containing VP2, VP6 and VP7. Therefore, these three structural proteins are described below in more detail.

VP2. The innermost protein layer of the rotavirus virion is composed of 120 copies of VP2 arranged as dimers. This layer surrounds the viral genome and RNA processing enzymes, including the RNA-dependent RNA polymerase VP1 and the capping enzyme VP3. The

particle structure at this level is referred to as the single-layer or core particle (93). Expression of VP2 in insect cells using recombinant baculoviruses resulted in assembly of VP2 proteins into core-like particles of 50 nm in diameter (56). The VP2 shell of rotavirus plays an important role in the structure and function of the core by interacting with trimers of VP6, which surround the VP2 layer and the shell is perforated with aqueous channels that transport nascent RNA out and metabolites in the core during transcription (127). The amino terminus of VP2 possesses a non-specific ss RNA and ds RNA-binding activity and is also essential for the incorporation of VP1 and VP3 into the core of the virion (57). Besides its scaffolding functions, VP2 also serves as cofactor for the activation of the viral polymerase VP1 to initiate the synthesis of viral ds RNA (72).

VP6. VP6 is the major structural component of virions forming the intermediate layer, and it plays a key role in the overall organization of the virion structure because of its interactions with both outer capsid proteins, VP4 and VP7, and the core protein VP2. Particles carrying VP6 on the outside are called double-layered particles and are the transcriptionally competent form of the virus (93). This middle shell is made of 780 copies of VP6, which spontaneously form trimers. Trimerization and the formation of tubules is an intrinsic property of the VP6 protein, since it forms these structures in the absence of other viral proteins (30). These trimers can be dissociated and reassembled by changing pH (58). VP6 is important as a structural component to maintain the proper conformation or organization of the particle. The VP2-VP6 interaction defines the correct geometry of the virion (69) VP6 is both highly immunogenic and antigenic, and it is the protein targeted most often in diagnostic assays as it contains common epitopes shared by other group A viruses. Co-expression of VP2 and VP6 from baculovirus vectors results in the formation of double-layered VLPs (20).

VP7. VP7 is the second most abundant capsid protein and the major constituent of the outer layer. 780 copies of this glycoprotein, grouped as 260 trimers, together with 60 spikes of VP4, form the outer-most layer of the virion. VP7 is reported to be highly immunogenic and is inducing neutralizing antibodies. VP7 is localized to the endoplasmic reticulum (ER) of the cell, where it is retained as a membrane-bound protein before assembly into maturing virion particles (3). VP7 is co-translationally glycosylated as it is inserted into the membrane of the ER, and insertion is directed by a cleavage signal sequence found at the amino-terminus of the protein. The nucleotide sequence predicts an ORF of 326 amino acids (aa), which begins with an initiation codon with a weak consensus sequence. A second in-frame initiation codon precedes two regions of hydrophobic aa (H1 and H2), which can act as the signal sequence to direct VP7 to the ER, although the second is thought to be the major species used in cells. A third in-frame initiation codon is also present downstream from the second hydrophobic

domain in some strains (29). Studies to determine the signals leading to the retention of VP7 to the ER have shown that VP7 does not contain the sequence KDEL found to confer retention for some other ER proteins. There are several studies reporting regions in the ORF responsible for retention of VP7 in the ER and also that these residues are critical for retention, but the method by which VP7 remains in the ER is unclear (65).

1.1.4 Rotavirus Lifecycle

RVs have a specific cell tropism *in vivo* and infect primarily the mature enterocytes of the villi of the small intestine. Entry of RVs into host cells is still poorly understood. Results from recent studies suggest that rotavirus cell entry involves a series of complex and coordinated events following proteolytic priming of the virus. The infectious virions (triple-layered particles TLPs) attach to the host cell through sialic acid containing receptors in the initial step. Next, interaction with integrins and Hsc70 occur during the subsequent post-attachment process (61). The spike-forming protein VP4 has essential functions in the virus life cycle, including receptor binding and cell penetration. The penetration of the plasma membrane is increased and most probably also dependent on trypsin treatment of the virus, which results in the specific cleavage of VP4 into VP8* and VP5* (62). This proteolytic cleavage enhances viral infectivity several fold (5). In the entry process, the VP8* domain is involved in the interaction with sialic acid-containing receptors, whereas VP5* is interacting with integrins. For some RV strains, involvement of sialic acid is not an essential step and viruses entering the cells in a sialic acid-independent manner are thought to mediate the entry step mainly by VP5* (18, 52).

The next step in the life cycle of RVs is the transcription of dsRNA segments into mRNA molecules that can be processed for viral protein production and template generation. Replication takes place exclusively in the cytoplasm. During the entry process, the outer capsid shell of the TLPs is removed by cellular enzymes and a low intracellular Ca^{2+} level resulting in double-layered particles (DLPs). The DLPs are the transcriptionally active form and large numbers of positive-stranded RNA molecules (5' capped but not polyadenylated) are transcribed from all of the 11 RNA segments, which exit the DLPs via the 12 aqueous channels penetrating the VP2 and VP6 layers. The DLPs contain the full enzymatic machinery needed to synthesize the transcripts, to properly guanylate and methylate the cap structure of the mRNAs, and to facilitate the translation by the cellular translation machinery. VP1 is the RNA-dependent RNA polymerase (116) and VP3 is the guanylyl- and methyltransferase (13). The newly synthesized RNA molecules either act as mRNAs, leading to accumulation of the translated gene products in the cytoplasm, or undergo replication in intracytoplasmic inclusion bodies termed viroplasms. The two non-structural proteins NSP2 and NSP5 are the major components of viroplasms and have been shown to be sufficient to form these electron-dense cytoplasmic inclusions (33, 64). The synthesis of dsRNA and the

packaging of the RNA molecules into pre-virion core particles are taking place in the viroplasms (109). Besides NSP2 and NSP5, the viroplasms also contain VP1, VP2, VP3, VP6 and NSP6 and in the beginning all the transcribed mRNA from all the genomic segments (6, 40). At present, the exact order of events during early morphogenesis and the control mechanisms by which the packaging of the RNA segments into the cores occur are unknown. The DLPs formed in the viroplasm consist of VP1, VP2, VP3 and VP6 and contain one of each of the 11 ds RNA segments. Again, how the correct set of 11 segments of ds RNA gets encapsidated into each virion remains entirely unclear. One model suggests that the genomes are packaged concurrent with the capsid assembly (93).

Maturation and release are the final steps of the RV life cycle. Once the DLPs are formed, they bud from the viroplasms into the proximally located rough endoplasmic reticulum (rER) and receive a transiently rER-derived envelope layer, which is lost before complete maturation. During this step, the outer layer proteins VP4 and VP7 are incorporated. During budding of the DLPs through the rER, NSP4 acts as intracellular receptor for VP6 (7, 113). NSP4 and VP7 are both synthesized on the rER-associated ribosomes and co-translationally inserted into the ER membrane. Silencing the expression of NSP4 by siRNAs has been shown to block DLPs and TLPs, and the amount and distribution of the viral proteins was altered (63). Besides the role of NSP4 in viral morphogenesis, NSP4 is a viral enterotoxin capable of inducing diarrhea in mice on its own (8, 105) During the last step of RV morphogenesis, the transient lipid envelope is lost and the viral proteins are rearranged in order to form the outer layer consisting of VP7 and VP4, while NSP4 is excluded from the virus particle. The precise mechanism is not known, but the above mentioned siRNA experiments indicate that VP4 is assembled in the ER and VP7 is involved in the removal of the transient envelope (63). It was also reported that chaperones from the ER are involved in the quality control of the newly assembled virus particles (68).

In non-polarized cells, electron microscopy studies have shown that progeny virus is released by lysis of the host cell (2). Late during infections, there are drastic alterations in the permeability of the plasma membrane observed resulting in the release of cellular and viral proteins (80). The polarized intestinal Caco-2 cells have been shown to spontaneously display many of the morphologic and biochemical properties of mature enterocytes. In these cells, RV was released almost exclusively at the apical pole of the cells, before any cells lysis was detected. The RV transport pathway from the ER to the apical surface of the intestinal cells is described as a non-conventional vesicular transport of virus particles from the ER to the apical plasma membrane that bypasses the Golgi apparatus and lysosomes (49).

1.1.5 Current status of Rotavirus vaccines

The burden of RV disease is high worldwide. According to the estimates based on studies done worldwide during 1986 – 2000, RVs cause 100 million episodes of acute

gastroenteritis, 25 million clinic visits, 2 million hospitalizations and approximately 600'000 deaths per year (88, 89). The incidence of RV disease is similar for developed and developing countries, while deaths from RV infections are most frequent in developing countries of sub-Saharan Africa and Asia. RV-related deaths represent approximately 5% of all deaths in children younger than 5 years of age all over the world (27). Rates of RV illness in industrialized and less developed countries are similar, therefore, the supply of clean water and good hygiene have little effect on virus transmission. This highlights the urgent need for safe and effective RV vaccines, particularly in developing countries where timely healthcare is not always available and mortality because of RV infections is high (26).

Human RVs show enormous diversity as the gene segments that encode the G (VP7) and P (VP4) proteins can segregate independently, giving rise to strains with at least 42 different P-G serotype combinations (39). However, there are a small number of prevalent RV strains representing over 88% of the analyzed strains worldwide. The G and P serotypes also differ geographically, meaning that an effective vaccination program should take into account this geographical variation of the prevalent strains. Also monitoring the circulating strains after introduction of vaccine candidates may be necessary, as vaccine pressure may lead to the selection of novel RV strains.

The first vaccine programs started in the early 1980s and these approaches were based on the "Jennerian" concept. This concept is using live attenuated animal RV strains (of simian or bovine origin) for the immunization of infants, as these strains are considered to be naturally attenuated in humans. None of these vaccine candidates however induced protection from severe RV disease, which led to the next generation of vaccines. Instead of using only one RV strain (monovalent vaccine), vaccines based on the "Modified Jennerian" approach, using human/animal RV reassortants, have been generated. This approach was based on the fact that RVs are able to reassort during double infections. These live reassortant virus vaccines contain some genes from the animal RV parent and some from the human RV parent. In general, the selected RV reassortants contained the VP7 gene derived from the human RV parent, as this protein was thought to be important for protection. The remaining genes were from the animal RV strain, giving a genome background of a non-pathogenic animal RV expressing the human P and G types. The first multivalent live oral reassortant vaccine developed was RotaShield, a rhesus rotavirus (RRV) tetravalent vaccine. It contained the VP7 gene derived from human RV strains belonging to the G types, G1, G2 and G4. As RRV belongs to the G3 type, this vaccine represented the four epidemiologically most important RV G serotypes. Protective efficacy of this vaccine against severe RV disease was 70 to 100% in large field trials; therefore, it was recommended for universal immunizations in the US and licensed in 1998. After vaccinating over 600'000 children in the US, several cases of vaccine-associated intussusception (the invagination of the intestine into a distal segment)

were reported and its manufacturers immediately withdrew Rotashield from the market. Initially, VP7 was thought to be the most important antigen in inducing protection, but more recently, also VP4 was considered to be important for protection. Therefore, human-animal reassortant RV vaccine candidates now include either human VP7 or VP4 genes (10, 26, 27, 92, 108).

Currently licensed RV vaccines. (4, 28, 120). The first RV vaccine after the withdrawal of RotaShield in 1999 was licensed in Mexico in 2004. This attenuated human vaccine Rotarix became available in January 2005 and was produced by GlaxoSmithKline. Since then, this vaccine has been licensed in nearly 100 countries, including European countries. The monovalent (contains a single human RV strain) vaccine is derived from a human RV isolate and attenuated by multiple passages in tissue culture. This vaccine was generated on the assumption that one natural RV infection in children prevents a second severe infection. Rotarix was evaluated in several studies and found to be highly efficient (85%) in preventing severe RV gastroenteritis and hospitalizations. Because of the association of RotaShield with intussusception, the next vaccines had to undergo large safety trials. Rotarix was evaluated in a study with over 63'000 infants and shown to be safe and, most importantly, not associated with intussusception (97). In 2006, the pentavalent reassortant vaccine Rotateq, developed by Merck, was licensed, first in the USA and subsequently also in the European Union. This RV vaccine is based on the attenuated bovine RV strain WC3. It consists of a mixture of five mono-reassortant strains, each of which contains a gene encoding for VP4 or VP7 from a RV of human origin, but the other segments in all mono-reassortants are provided by the bovine WC3 strain. Therefore, this vaccine was generated to contain most of the different serotypes that a child will be exposed to in the assumption that this is the most effective approach to induce broad protective immunity. Also this RV vaccine underwent large safety trials, and it was found to be highly effective in preventing severe RV gastroenteritis (98%), and no link with intussusception was found.
In Switzerland, vaccination against RV is not included in the national vaccination schedule, as there are not many deaths caused by RV infections (BAG and EKIF 2008) and the disease is efficiently treated by rehydration. Nevertheless, Rotarix is licensed in Switzerland. There are an estimated 6200 doctoral visits and 500-1000 hospitalizations due to RV disease reported each year in Switzerland, but the vaccines are too expensive (approx 216 CHF) relative to the expected benefit.

Alternative RV vaccine candidates

All currently licensed RV vaccines are live, orally administered vaccines that aim to mimic the protection given by naturally occurring RV infections, and all RV vaccines tested in children

have been either live viral vaccines of a Jennerian or modified Jennerian approach or attenuated human RVs.

The original expectation for a successful RV vaccine was that it should protect against almost all cases of RV disease of any severity. After realizing that even natural infections with RV do not provide complete protection against subsequent RV illnesses, a more realistic goal for a RV vaccine would be that it provides a high level of protection against severe RV diseases. The currently licensed vaccines realized this goal so far, however, none of the efficacy trials have been completed in the Third World nations where deaths because of RV infections are most common and where the earlier RV vaccine candidates failed to provide significant protection. Together with not fully resolved safety concerns associated with live vaccines, non-living RV vaccine candidates are developed as possible next generation vaccines. These candidates range from fully intact, inactivated RVs to pieces of RV proteins and even DNA vaccines. However, three types of these next generation vaccine candidates are most promising. These include inactivated triple- and double-layered RV particles, triple- and double-layered virus-like particles (VLPs) and recombinant, *E.coli*-expressed VP6 proteins. Since the 1980s, these vaccine candidates are being tested in animal models, but until now, none have been evaluated in humans, not even in phase 1 trials (119).

Inactivated virus. Viral particles are inactivated by physical or chemical methods and delivered by either parenteral or mucosal (intranasal) routes. This approach was tested in mice (75, 76), rabbits (19) and gnotobiotic piglets (114, 126) and found to be effective in reducing or preventing RV shedding with or without the use of adjuvants. Parenteral immunization with formalin-inactivated RV strain RRV combined with lipid adjuvants was evaluated in the infant mouse model and showed to protect the offspring from clinical diarrhea upon challenge (47).

Virus-like particles (VLPs). Production of RV VLPs was first reported in the 1980s (20, 32, 94). There are many expression vectors to produce the recombinant proteins for VLPs formation, but the well-established baculovirus system is the most common expression vector used and also facilitated the analysis of virus structure and, to some extent, of virus assembly. Core, double- and even triple-layered VLPs have been produced in insect cells infected with baculovirus vectors (20, 29, 55). However, the limitations of the baculovirus system include the inefficient infection of mammalian cells and, consequently, the need to purify VLPs from infected insect cells for vaccination. Insect cells may not be suitable to study virus assembly either, because the posttranslational processing of proteins in insect cells greatly differs from that in mammalian cells (54, 79). Therefore, viral vectors that can infect mammalian cells, including Semliki Forest virus based replicons (81) and vaccinia virus

vectors (41), have also been evaluated for the generation of rotavirus VLPs. Some VLPs contain only VP2 and VP6, while others have incorporated one or more of the neutralization proteins VP4 or VP7; some VLPs are also composed of the full set of the structural proteins. The use of a VLP prime (VLPs containing VP2 and VP6) and recombinant adenovirus expressing VP6 boost regimen were found to elicit stronger humoral, mucosal and cellular immune responses and confer stronger protection against RV challenge than each of the VLPs alone (128). The effect of maternal antibodies on different routes of administration was investigated using non-replicating RV VLPs of bovine origin composed of VP2, VP6, VP7 and VP8* fused to VP2 and both, parenteral and oral immunization of newborn mice resulted in specific serum IgG response (48).

***E.coli*-expressed VP6 proteins.** As VLPs composed of only VP2 and VP6 provided excellent protection against RV shedding when they have been administered intranasally with a powerful adjuvant (84), it was possible that the VP6 protein alone could stimulate protection against RV shedding. Therefore, *E.coli*-expressed VP6 proteins from murine or human RV strains have been tested in mice. They were orally, intrarectally or intranasally administered together with effective adjuvants and found to effectively protect mice against fecal RV shedding (17). In another approach, VP6 was expressed in *E.coli* as a fusion protein with maltose binding protein and administered intranasally to mice together with a potent adjuvant. After challenge, the mice were protected nearly 100% from fecal shedding of RV (15, 16). VP6 is the group antigen and therefore highly conserved in group A RVs, thus it is not surprising that protection of mice immunized with VP6 from a human strain was highly effective when mice were challenged with heterotypic murine RVs.

Is there need for another RV vaccine?

Taken together, several drawbacks have affected the development and application of the RV vaccines: (i) In general, RV vaccines have performed better in developed than in non developed countries, probably due to inhibition by higher natural exposition before vaccination, or atypical serotypes prevalence in less developed countries (10). (ii) The withdrawal of RotaShield because of the observed association with intussusception resulted in extended testing and delays in the licensure of the next vaccine candidates. (iii) Another difficulty is the rapid generation of the specific reassortants, making it complicated to adapt the vaccine for use in regions where the dominant serotype is not included in the original formulation (i.e. G5 in Brazil, G9 in Argentina, etc). (iv) At least three doses are necessary to reach adequate protection against all the included serotypes, which makes vaccination difficult, expensive, or impracticable. Also the young age of administration because of the strict safety settings from the clinical trials will be extremely difficult to achieve in developing

countries. (v) The oral delivery implies that special buffer and stabilization components should be added to the formulation in order to avoid inactivation by the stomach milieu. (vi) One main concern is based on the constant and rapid evolution of RV, due both to their RNA genome and to the fact that their genes can reassort between strains co-infecting the same host. This fact is an important threat to the rational of using live attenuated rotavirus strains as vaccines, both because these strains can revert to more virulent phenotypes and because these vaccine strains are being released to the nature in the faeces of the inoculated persons, raising a considerable ecological concern (51).

1.2 Herpesviruses

Herpesviruses are highly disseminated in nature and most animal species have yielded at least one herpesvirus upon examination (90). The word *herpes* means to creep or crawl, in reference to the spreading nature of the visualized skin lesions that have been documented since ancient Greek times. The membership in the family *Herpesviridae* is based on the architecture of the virion. A typical herpesvirion consists of a core containing a linear double-stranded DNA, an icosadeltahedral capsid of approximately 100-110 nm in diameter, the tegument that surrounds the capsid, and an envelope containing viral glycoprotein spikes on its surface (Figure 3). The family contains more than 130 members with a host range from mammals and birds to amphibians and reptiles.

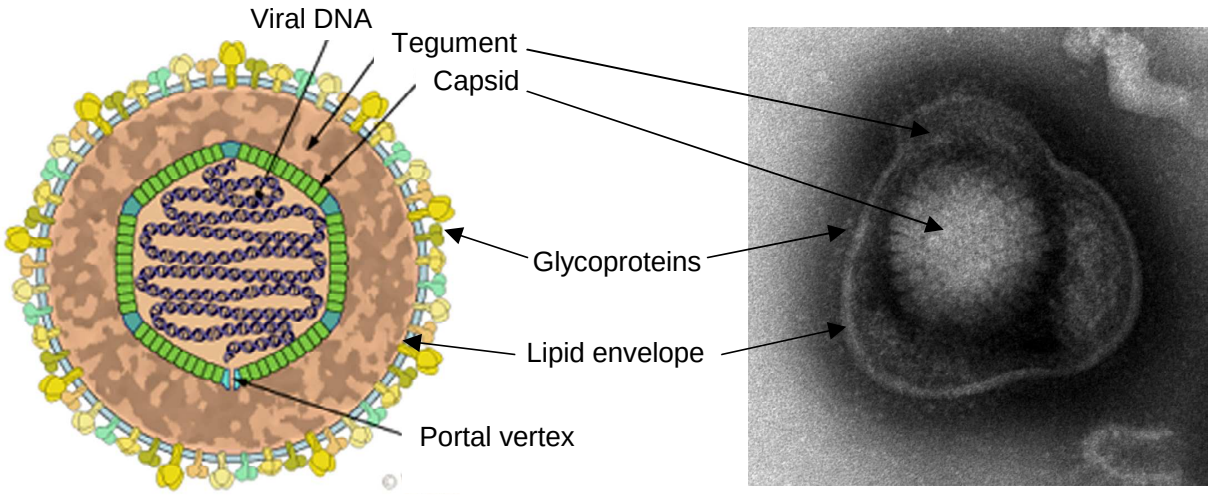

Figure 3: HSV-1 virion structure. The major structural components of the HSV-1 virion are: the viral dsDNA packaged in an icosahedral capsid, the tegument layer containing multiple viral proteins and the lipid membrane envelope studded with viral glycoproteins. On the right: electron micrograph (Negative contrast, E. Schraner University of Zurich). Schematic of a HSV-1 virion is shown on the left (Adapted from ViralZone: www.expasy.org/viralzone, Swiss Institute of Bioinformatics).

The known herpesviruses appear to share four significant biologic properties (90): (1) All herpesviruses specify a large array of enzymes involved in nucleic acid metabolism, DNA synthesis and processing of proteins, although the exact array of enzymes may vary from one herpesvirus to another. (2) The synthesis of viral DNAs and capsid assembly occurs in

the nucleus and capsids are enveloped as they transit through the nuclear membrane. (3) Production of infectious progeny virus is invariably accompanied by the destruction of the infected cell, but the infected host is in general not killed. (4) The herpesviruses examined to date are able to remain latent in their natural hosts. Latency occurs in the natural host and in specific cell types, and many herpesviruses have specific genes required for the establishment of latency. In the latent state, the viral genome is present as a circular episome that replicates along with the DNA of the host cell and only a small subset of viral genes are expressed, and there is no production of infectious virus. Latent genomes retain the capacity to replicate and cause disease upon reactivation that is dictated by the environment of the host cell. The precise mechanisms that lead to reactivation from the latent state are not fully understood and may differ from one virus to another. Herpesviruses also differ with respect to their biologic properties. Some have a wide host-cell range, multiply efficiently and rapidly destroy the cells they infect. Others have a narrow host cell range or have a long replicative cycle. The members of the family *Herpesviridae* were classified into three subfamilies on the basis of biologic properties and before DNA sequences of the individual members were known: α-, β- and γ-*Herpesvirinae*. The members of the subfamily *Alphaherpesvirinae* were classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells and the ability to establish latent infections primarily but not exclusively in sensory ganglia. This subfamily contains the genera *Simplexvirus* (HSV-1), *Varicellovirus* (VZV), *Mardivirus* and *Iltovirus*. The first two genera have mammalian hosts, whereas the latter two have avian hosts (90).

1.2.1 Life cycle of herpes viruses

To initiate infection, the virus must attach to cell surface receptors. This initial attachment rapidly follows the transmission of the virus through the plasma membrane. The de-enveloped tegument-capsid structure is then transported to the nuclear pores, where DNA is released into the nucleus. Transcription of the viral genome, replication of viral DNA and assembly of new capsids take place in the nucleus, whereas all viral proteins are synthesized in the cytoplasm. During productive infection, the viral proteins are expressed in a highly regulated cascade fashion in a number of co-ordinately expressed groups of gene products, and several proteins play a role in regulation of viral gene expression. The host RNA polymerase II is responsible for transcription of all viral genes during infection, although viral gene products may modify its activity and structure. Virus entry is followed by immediate-early and early gene expression. Several of the early gene products are enzymes and DNA-binding proteins involved in viral DNA replication. The bulk of viral DNA is synthesized by a rolling-circle mechanism, yielding concatemers, which are cleaved into monomers during the process of capsid assembly. The viral proteins involved in replication of

the viral DNA and nucleotide metabolism promote viral DNA replication, which in turn stimulates the expression of late, genes. The ultimate goal of viral late gene expression is to produce large amounts of viral structural proteins for assembly of progeny viral particles. Viral capsid assembly occurs in the infected cell nucleus and, after encapsidation of full-length viral genomic DNA molecules, the virus matures, acquires infectivity and is now able to spread from one cell to another.

1.2.2 Herpes simplex virus type 1

Herpes simplex virus type 1 (HSV-1) is a common human pathogen causing infections of orofacial mucosal surfaces. Productive infections lead to the formation of vesicular lesions in the mucosal epithelia, which is followed by spread of the virus to sensory neurons and establishment of a latent infection. This latent state may remain for the lifetime of the infected host and reactivation of lytic infection results in recurrent disease at or adjacent to the site of the primary infection. The cold sores caused by HSV-1 are not life-threatening, but the virus can infect the central nervous system, leading to serious diseases like keratitis and encephalitis (121). The virus can efficiently infect a wide variety of cells, including dividing and non-dividing cells.

HSV-1 is a large (150-200 nm in diameter) enveloped virus composed of three different compartments, an icosahedral capsid containing the dsDNA genome, the tegument that contains additional viral proteins and an envelope of host origin equipped with viral glycoproteins (Figure 1). The genome is composed of 152 kb linear double-stranded DNA encoding at least 80 genes (Figure 5). Two covalently linked segments are forming the genome, both segments contain unique regions (unique long U_L and unique short U_S) each flanked by inverted repeats (terminal and internal). HSV-1 contains three lytic origins of replication, named depending on their location, *ori*$_L$ or *ori*$_S$, and two DNA packaging signals, *pac*, which ensure proper cleavage and packaging of unit-length progeny genomes (25, 95).

The understanding of the biological properties of HSV-1 and the molecular mechanisms of virus replication has allowed the development of potential vectors for several applications in human healthcare, including delivery and expression of human genes to cells of the nervous system, selective destruction of cancer cells, prophylaxis against infections of HSV or other infectious diseases and targeted infections of specific tissues or organs. Three different types of vectors can be derived from HSV-1: (1) Replication-competent attenuated vectors carrying mutations, which restrict spread and lytic viral replication to the specific target cells. These vectors are being used mainly as oncolytic viruses. (2) Replication-incompetent defective recombinant vectors, which either lack or are deleted for one or more genes essential for in vitro virus replication. (3) Defective helper-dependent vectors known as amplicons, which carry no viral genes and take advantage of the large transgene capacity of the HSV-1 particle (23, 67, 112).

1.2.3 HSV-1 amplicon vectors

HSV-1 amplicon vectors originated about 30 years ago when Spaete and Frenkel (110) analyzed the nature of defective virus genomes generated during passage of standard HSV-1 stocks at high multiplicity of infection. Their investigations revealed that an origin of replication and a cleavage/packaging signal, pac, were the only two *cis*-acting sequences required for the replication and packaging of defective virus genomes in the presence of *trans*-acting HSV-1 helper virus (111).

Amplicon vectors are HSV-1 particles identical to wild-type HSV-1 from the structural, immunological and host-range points of view, but instead of the viral genome, they carry a DNA plasmid in a concatemeric form, named the amplicons plasmid. The HSV-1 amplicon particles are composed of approximately 40 different virus-encoded structural proteins, which are delivered into the cell during infection. Therefore, they can trigger cell signalling and cellular responses and may have a transient impact on cell homeostasis or gene expression, but these proteins will soon disappear and the cells retain their normal functions (21, 115).

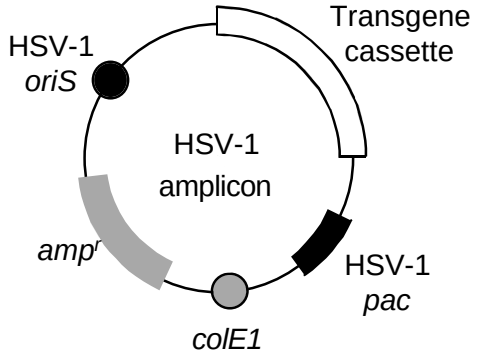

Figure 4: Basic HSV-1 amplicon plasmid. The two non-coding elements of HSV-1, the origin of replication *ori*$_S$ and the packaging/cleaving signal *pac* allow replication of amplicon DNA and packaging into HSV-1 virions. For the propagation in *E.coli*, the plasmid contains an origin of replication (*colE1*) and the antibiotic resistance gene (*amp*^{r}). The transgene cassette usually contains a fluorescent marker to titrate the amplicon vector stocks.

Current HSV-1 amplicon plasmids are standard *E.coli* plasmids carrying typically two non-coding elements of HSV, *ori*$_S$ and *pac*; a bacterial origin of replication and an antibiotic-resistance gene for propagation in *E.coli*; a fluorescence marker gene and the transgene expression cassette with the gene(s) of interest (Figure 4). Amplicons can easily be constructed by recombinant DNA technologies, shuttled between prokaryotic and eukaryotic cells and packaged in different cell lines as a single amplicons of up to 150 kb or as concatenates with multiple copies of the transgene cassette into HSV-1 virions (85) due to the rolling circle mode of HSV-1 DNA replication and the genome packaging capacity of the HSV-1 capsid (~150 kb). This results in very high levels of gene expression (106). One major advantage of the amplicons vectors as gene transfer tools is the fact that they do not carry viral genes and consequently do not induce synthesis of HSV-1 proteins. Therefore, these vectors are entirely non-toxic for the infected cells and non-pathogenic for the transduced organism; reactivation, complementation or recombination with latent or resident HSV-1 genomes is strongly reduced, especially when amplicon vectors are generated using a helper-virus free system (99). HSV-1 amplicons do not express any viral genes but depend

on helper functions for replication and packaging into HSV-1 virions. For the production of amplicon vector stocks, essential HSV-1 functions are supplied *in trans* by a viral genome or a helper virus. Currently, two *trans*-complementing systems are used, based either on cloned HSV-1 DNA or on helper HSV-1. Both systems can generate contaminating helper HSV-1 particles, which are toxic for the cells and can induce inflammation, which is the main concern to use these vectors in gene therapy (21).

Helper virus-dependent production of HSV-1 amplicon vectors

The helper virus-assisted packaging system developed by Epstein and co-workers is a two-step process with an initial co-replication and packaging of helper virus and amplicon plasmids. The packaging signal of the helper virus is deleted by Cre/loxP-based site-specific recombination, allowing preferential packaging of the amplicon DNA, which contains the *pac* signal. The helper virus contains a single *pac* signal flanked by two *lox*P sites in parallel orientations and a deletion of the two genes surrounding the *pac* signal, encoding a virulence factor ICP34.5 and the essential protein ICP4. The amplicon vector stocks are produced in a first step in ICP4-complementing cells transfected with the amplicon plasmids and infected with helper virus, resulting in a mixture of amplicon vectors and helper virus. In a second step, this mixed stock is used to infect ICP4 and Cre-expressing cells, where Cre recombinase mediates the excision of the *pac* signal from the helper genome, inhibiting helper virus packaging. With this system, large vector stocks can be prepared, containing around 0.05 to 0.5% of contaminating helper virus. As ICP4 is deleted in the helper genome, this helper virus cannot replicate in non-complementing cells (21, 60). Nevertheless, the contamination with helper virus, although defective, may be toxic to sensitive cells, and may evoke an immune response to transduced cells; recombination with endogenous HSV-1 may also occur.

Helper virus-free packaging of HSV-1 amplicon vectors

Helper virus-free packaging of amplicon vectors has undergone continuing improvement (Figure 5). The initial system was based on transfection of a set of five overlapping cosmids encoding the entire HSV-1 genome to provide the necessary functions for the replication and packaging of amplicon DNA into HSV-1 particles. The packaging of the HSV-1 genome itself was blocked by deletion of the DNA cleavage/packaging (*pac*) signals from the cosmids (22, 36). Limitations to this amplicon packaging include (i) the genetic instability of the cosmid clones, (ii) complicated procedure in preparing five HSV-1 DNA fragments from cosmids and (iii) difficulty in large scale production of vector stocks (106). To overcome the first two problems, the entire *pac*-deleted HSV-1 genome was subcloned into a single-copy F-plasmid-based bacterial artificial chromosome (BAC), fHSVΔ*pac* (100). This BAC-based

packaging system achieves several improvements over the cosmid-based version, including (i) genetic stability of the helper HSV-1 genome in *E.coli*, (ii) a simplified packaging procedure and (iii) increased amplicon vector titers. There was still the possibility of a homologous recombination event between the ori_s sequences present on both fHSVΔpac and amplicon, thus allowing reintroduction of *pac* into the helper genome resulting in contamination of the vector stocks with replication-competent helper virus. Virtual elimination of contaminating replication-competent helper virus has now been achieved by deleting an essential HSV-1 gene, ICP27, from fHSVΔpac and providing ICP27 in *trans* from another plasmid (98, 99).

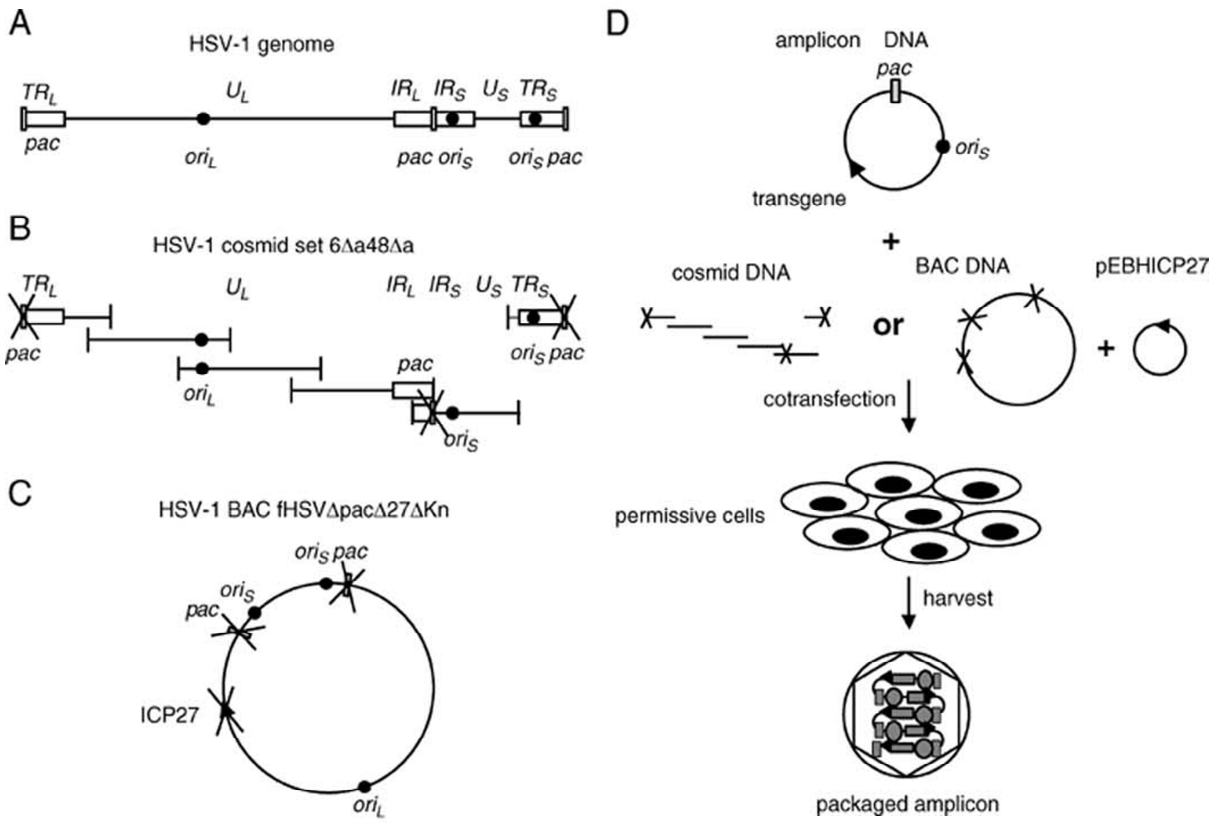

Figure 5: Helper virus-free packaging system. (A) The HSV-1 genome is formed of two covalently linked segments, both segments contain unique regions (unique long U_L and unique short U_S) each flanked by inverted repeats (terminal TR and internal IR). HSV-1 contains three lytic origins of replication, named depending on their location, ori_L or ori_S, and two DNA packaging signals (*pac*) (B) Schematic diagram of HSV-1 cosmid set with deleted (X) packaging signals (*pac*). (C) Schematic diagram of HSV-1 helper BAC DNA. This plasmid contains all the *trans*-acting functions required for replication and packaging of the amplicon DNA, but lacks the *cis*-acting elements required for its own packaging (*pac* signals) and the ICP27 to eliminate possible recombination. (D) DNA based method for amplicon vector production. Co-transfection of permissive cells with amplicon plasmid and either cosmid DNA (left) or BAC DNA and ICP27-expressing plasmid results in helper virus-free amplicon stocks. (From Current Protocols in Neuroscience 2007; with permission (35))

The current packaging system is based on this oversized *pac*- and ICP27 deleted HSV-1 genome cloned as a BAC (fHSVΔpacΔ27). This system requires co-transfection of amplicon

DNA and the HSV-1 helper DNA into permissive cells, the VERO 2-2 cell line (Figure 5). This cell line is stably transfected with the ICP27 gene. Complementation of the ICP27 gene is further achieved by co-transfection of the cells with an ICP27-expressing plasmid. Using this helper BAC, fHSVΔpacΔ27, with the deletion in ICP27, amplicon vectors are packaged without detection of replication-competent virus and are therefore entirely helper virus-free.
However, the amount of total amplicon vectors produced by this method is limited, as these vector stocks cannot be further amplified. Another reason why the helper virus-free system results in lower vector titers may be due to viral tegument proteins which are present in the helper virus and are known to prime the cell for virus production via the trans-activation of immediate early genes (VP16) or by regulating cellular and viral transcription (vhs) (24). However, further optimization and refinement of the currently used helper virus-free systems will be required to achieve titers high enough for clinical testing of the amplicons vectors.

1.2.4 HSV-1 amplicons as vaccine vectors

Infection with wild-type HSV-1 results in a potent host immune response involving both, the innate and the adaptive immune system (53), but the virus is also able to evade the host immunity through a variety of mechanisms based upon immuno-modulatory gene products such as the virion host shutt-off protein, *vhs* (102), or the ICP47 protein (125). HSV-1 amplicon vectors are very promising candidates for vaccine delivery because they retain the strong inherent immunogenicity of the parental virus, while eliminating many of the unwanted viral functions for immune evasion. Other characteristics of the amplicon vectors include their safety, large transgene capacity and the wide host range.
It has been demonstrated in a number of studies, that HSV-1 amplicon vectors are able to transduce antigen-presenting cells (APC) without impairing their antigen-presenting functions. Using helper virus-free amplicons, it was shown that about 70% of bone marrow derived murine dendritic cells (DC) were transduced without loss of function (122). Two other groups documented efficient expression of model transgenes by transduced DCs without impairment of the antigen-presenting function (42, 83), which is an important task for the use of amplicons vectors as vaccines. Most studies focused on the immunization against HIV. One group showed that a single dose of $1x10^6$ transducing units of amplicon vectors expressing the HIV envelope glycoprotein gp120 was able to elicit strong, antigen-specific and long-lasting cellular and humoral responses in mice (44). Subsequent work showed that amplicon-induced immune responses could strongly be enhanced by prior priming with either a DNA vaccine encoding the same antigen or by an initial prime with the amplicon vectors (118). Taken together, amplicon vectors have shown promising results as vaccine delivery system in several preclinical systems, including animal models for cancer treatment and prophylactic immunizations against infectious pathogens (HIV-1). Another often discussed difficulty is the effect of pre-existing immunity on HSV-1 mediated gene transfer, as HSV-1 is

an ubiquitous virus in the human population and, thus, anti-HSV-1 antibodies are present in most adults. This may interfere with amplicon-mediated vaccination, as these could lead to a direct vector neutralization or rapid clearance of the amplicon-transduced cells. There is one study where the investigators used a heterologous HSV-1 strain to generate an anti-HSV-1 immune response in mice, which were subsequently immunized via the subcutaneous route using helper virus-free amplicons vectors. The mice showed significant titers of neutralizing antibodies, but this resulted in only a modest reduction in the amplicon-induced cellular response (44). These data suggest that the pre-existing immunity in hosts may not prevent the successful use of amplicon-based vaccines.

1.3 Virus-like particles

The structural proteins of many viruses have the ability to self-assemble spontaneously into particles that are similar to the authentic viruses. These virus-like particles (VLPs) are non-replicative and non-pathogenic because they assemble without incorporating genetic material, thus they do not contain any viral genome. The term “virus-like” refers to the morphological similarity of the VLP to the corresponding virion. Depending on the complexity of the virus, VLPs can be produced from one or several structural proteins being in the general size range of the parent virus, with their exact size and morphology depending on the particular viral proteins they are made of (82). VLPs are structurally stable and can be manipulated to carry and display heterologous molecules. Multiple capsid proteins may be assembled either from expression and subsequent processing of a precursor protein or by co-expression of the capsid proteins from multicistronic vectors in the same cell (117). There are many expression systems for the production of VLPs, including (i) various mammalian cell lines, either transiently or stably transfected or transduced with viral expression vectors (74, 103, 123); (ii) the baculovirus/insect cell system (37, 45, 129); (iii) various species of yeast (38, 71, 104) and (iv) *Escherichia coli* and other bacteria (59, 78, 124). The choice of the production system depends on the type of VLPs to be produced and the number of different structural proteins the VLPs are made of. Mammalian cell culture systems are favoured because they support appropriate modifications and authentic assembly, but are less controllable and more costly for production, whereas the *E.coli* system may be efficient, but often there are additional steps required for the assembly (14). Clearly, not all VLPs generated to date are used as vaccines, some have been generated to understand the assembly process, study the morphogenesis or the architecture of the viruses.

Virus-like particles for vaccination

The most potent vaccines used in the past are attenuated or inactivated forms of whole viruses, but there always remains the risk of reversion into the pathogenic virus. VLPs on the other hand are safe as they do not contain infectious genetic material. Safety is the most

important requirement for vaccines today, especially when they are used for prophylaxis. Therefore, it is crucial to generate vaccines that are safe but at the same time able to induce potent and long-lasting immune responses. VLPs offer a promising approach to the production of vaccines against many diseases, because they contain well-exposed, repetitive domains on their surfaces, leading to the display of highly ordered or even conformational epitopes, which is often effective in eliciting strong immune responses (43, 107). VLPs are generally more immunogenic than subunit or recombinant protein immunogens, do not need immune stimulating adjuvants and are able to stimulate both the humoral and cellular arms of the immune system, including neutralizing antibodies (91, 96). The fact that VLPs are closely similar to the equivalent virus made them most promising candidates for the development of vaccines against the corresponding virus. A wide variety of VLPs have shown promising results in small animal models and may offer great potential for the development of vaccines. To date, two vaccines for humans based on VLPs are licensed, the papilloma virus vaccine and the hepatitis B virus vaccine (46, 73). Several vaccines based on VLPs are in development and in clinical trials, including vaccines against hepatitis C, malaria, SARS, Ebola, Marburg virus, Rift Valley fever, influenza, Norwalk virus, HIV and rotavirus (43, 86). However, there is a major limiting factor to overcome for the successful introduction of more VLPs-based vaccines, this are the high production and purification costs to obtain the large amount of highly purified particles needed for large clinical trials. Vaccination using VLPs is costly compared with other vaccines, which makes it difficult to perform clinical trials.

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2 Results

2.1 Results Part I: Rotavirus

2.1.1 HEVAR

Herpes virus-based vaccines against rotavirus infections (HEVAR) is a collaborating European project involving laboratories from Europe and South America. The overall scientific goal of HEVAR was to contribute to a better understanding of the immune biology of rotavirus infections using a novel generation of gene transfer vectors derived from herpes simplex virus type 1 (HSV-1).

One goal of this project was to construct HSV-1 based vaccine vectors expressing the structural genes of rotavirus using the helper virus-free HSV-1 based amplicon vector system. These antigens are expressed either individually or in different combinations, allowing in the most complex case the *in situ* generation of empty rotavirus like particles (VLPs) inside the vector-vaccinated organisms. Furthermore, the capability of these vectors to elicit protective immune responses is tested in mice.

2.1.2 Aim of the project

Rotaviruses are the cause of life-threatening gastroenteritis in children worldwide, thus this global and enormous disease burden has led to the effort to develop safe and effective vaccines.

The goal of this thesis was to construct and evaluate HSV-1 amplicon vectors encoding individual or multiple structural rotavirus proteins from a polycistronic transgene cassette. In this particular case, the amplicon vectors express two or three structural genes forming the double- or triple-layered capsid of the rotavirus, respectively. The capability of the vectors to support the in situ production of rotavirus-like particles in vector-infected mammalian cells is demonstrated. Furthermore, the potential usefulness of these amplicon vectors for vaccination is evaluated by assessing their ability to induce specific antibody responses in vector-vaccinated mice and to protect from disease upon challenge with wild-type rotavirus.

2.1.3 Manuscript Rotavirus

Own contribution to the manuscript:

Construction and characterization of HSV-1 amplicon vectors (IF and WB)

Production of vector stocks for immunization of mice

Characterization of VLPs by electron microscopy (assistance from E. Schraner)

Analysis of VP6 splicing

Writing of the manuscript

HSV-1 amplicon vectors launch the in situ production of rotavirus-like particles and induce rotavirus-specific immune responses in mice

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Abstract

Virus-like particles (VLPs) are promising vaccine candidates because they represent viral antigens in the authentic conformation of the virion and are therefore readily recognized by the immune system. As VLPs do not contain genetic material they are safer than vaccines produced with attenuated viruses. In this study, herpes simplex virus type 1 (HSV-1) amplicon vectors were constructed to co-express the rotavirus (RV) structural genes VP2, VP6, and VP7 and were used as platforms to launch the in situ production of rotavirus-like particles (RVLPs) in vector infected cells. We demonstrate that these vectors indeed supported the efficient production of RVLPs in infected mammalian cells. Intramuscular injection of mice with the amplicon vectors as a two-dose regimen without adjuvants resulted in detectable humoral immune responses. Moreover, partial protection was observed in immunized mice challenged with wild type RV strain EC, as shown by significantly reduced shedding of RV antigen in stool. This work provides proof of principle for the potential usefulness of the HSV-1 amplicon vectored production of RVLPs as genetic vaccine.

Introduction

Rotaviruses (RVs) are segmented, double-stranded (ds) RNA viruses of the *Reoviridae* family and are the most common cause of acute viral gastroenteritis in infants and young animals around the world. Almost all children both in developing and developed countries are infected with RVs during their first years of life and even advanced levels of sanitation and hygiene appear unable to control the spread of RV infections. Death from RV infection is most prevalent in developing countries where timely health care is not always available, causing more than 600,000 deaths per year (17, 101). Although the recently licensed human RV vaccines, which are based on orally administrated live attenuated strains, are very successful, data from clinical trials and post-licensure studies indicate that both vaccines are significantly less effective in low-income countries of Africa, Asia and Latin America (73, 77, 97). Additionally, potential safety issues like the risk of intussusception, inadvertent immunization of immunosupressed individuals and generation of new pathogenic strains by reassortment of vaccine strains with wild-type human and animal RV, suggest that development of new RV vaccines is still needed. Owned to the history of lower efficacy of all live oral vaccines in low-income countries, alternative approaches like parenteral vaccines should be pursued (44). Among these, inactivated RV particles, VLPs, subunit and vector based vaccines have been tested in animal models (9, 47, 108).

Mature infectious RV particles are non-enveloped, triple-layered icosahedral capsids. The innermost layer, composed of VP2 protein, encloses the 11 genomic segments of dsRNA. The middle layer is composed of the major capsid protein VP6, and the outermost layer is made of the glycoprotein VP7 and spikes of VP4. Both outer proteins are targets for neutralizing antibodies and define the virus G and P serotypes (38). Although a component of the middle layer in the mature particle, VP6 elicits a strong humoral immune response, and at least one strong T-cell epitope has been mapped which is highly conserved in most group A RV strains (2). The viral genome encodes six structural proteins (VP1 to VP4, VP6, VP7) and, depending on the viral strain, five to six nonstructural proteins (NSP1 to NSP6) (22).

The structural proteins of many viruses have the ability to assemble spontaneously into particles that are similar to the authentic viruses. These virus-like particles (VLPs) are in the general size range of viruses, with their exact size and morphology depending on the particular viral proteins they are made of (69). Importantly, VLPs are replication-defective because they assemble without incorporating genetic material. VLPs offer a promising approach to the production of vaccines against many diseases, because their repetitive, high density display of epitopes is often effective in eliciting strong immune responses (34, 89). VLPs are generally more immunogenic than subunit or recombinant protein immunogens and

are able to stimulate both the humoral and cellular arms of the immune system (74, 76). VLPs provide the spatial structure for display of conformational epitopes and, in doing so, are most likely to mimic the native virus structure, thereby enhancing the production of neutralizing antibodies. A wide variety of VLPs have shown promising results when applied in small animal models and may offer great potential for the development of vaccines (34). To date, two VLP-based vaccines are licensed for application in humans, the papilloma virus vaccine and the hepatitis B virus vaccine (42, 62).

Multiple capsid proteins may be assembled either from expression and subsequent processing of a precursor protein or by co-expression of the capsid proteins from multicistronic vectors in the same cell (98). There are many expression systems for the production of VLPs, including (i) various mammalian cell lines, either transiently or stably transfected or transduced with viral expression vectors (63, 82, 102); (ii) the baculovirus/insect cell system (27, 41, 109); (iii) various species of yeast (29, 61, 85) and (iv) *Escherichia coli* and other bacteria (55, 65, 105). Mammalian cell culture systems are favored because they support appropriate modifications and authentic assembly, but are less controllable and more costly for production.

The expression of RV proteins using the well established baculovirus system facilitated the analysis of virus structure and, to some extent, of virus assembly. Core, double- and even triple-layered RV-like particles (RVLP) have been produced in insect cells infected with baculovirus vectors (13, 20, 52). However, the limitations of the baculovirus system include the inefficient infection of mammalian cells and, consequently, the need to purify RVLPs from infected insect cells for vaccination. Also, insect cells may not be suitable to study virus assembly, because the posttranslational processing of proteins in insect cells greatly differs from that in mammalian cells (51, 67). Therefore, viral vectors that can infect mammalian cells, including Semliki Forest virus based replicons (68) and vaccinia virus vectors (30) have also been evaluated for the generation of RVLPs.

Herpes simplex virus type 1 (HSV-1) based vectors are being evaluated for gene therapy applications (7, 14, 37, 60, 71, 80) and are also considered promising vehicles to deliver antigens and immune-modulators for prophylactic and therapeutic vaccination, as well as for fundamental research (5, 14, 16, 43, 50). Specifically, HSV-1 amplicon vectors have a large transgene capacity (up to 150 kb), which allows the delivery of multiple transgenes or multiple copies of a single transgene. Additional advantages of HSV-1 amplicon vectors include (i) low toxicity and low immunogenicity, in particular when helper virus-free packaging systems are used (79, 88), (ii) high transduction efficiencies in dividing and non-dividing cells from most mammalian species, including antigen-presenting cells in vivo (83), (iii) genetic stability, and (iv) strong adjuvant effects, very long-lived immune responses, and the capacity of inducing both humoral and cellular immune response and mucosal immunity (36). HSV-1

amplicons have also been used for the synthesis of proteins from other viruses, e.g. amplicon vector mediated synthesis of the full set of structural proteins allowed the assembly of hepatitis C VLP (48, 95, 96) and retrovirus VLPs (72, 87). In particular, the possibility of inducing local assembly of inert VLPs in the context of a quasi-infectious process holds great promises as new vaccine formulation.

The goal of this study was to construct and evaluate HSV-1 amplicon vectors encoding individual or multiple structural RV proteins from a polycistronic transgene cassette in mammalian cells. Internal ribosome entry sites (IRES) in polycistronic vectors have been shown to support the expression of multiple genes from a single promoter (39, 66). As downstream cistrons are expressed at significantly lower levels (4, 18), the order of the RV genes was arranged within the polycistronic transgene cassette according to the relative abundance of the encoded protein in the virion. The expression of the RV genes was confirmed by Western blot and immune fluorescence analysis, and the generation of RVLPs in vector-infected cells was demonstrated by electron microscopy. Mice vaccinated with these vectors showed partial protection from challenge with wild type RV. These results demonstrate that polycistronic HSV-1 based amplicon vectors expressing genes for structural RV proteins are promising tools to study RV assembly in mammalian cells and may be useful as safe genetic vaccines against RV infections.

Material and Methods

Cells and viruses

African green monkey kidney epithelium, Vero (ATCC) and Vero 2-2 (90) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin G, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B, and, for VERO 2-2 cells, 500 µg/ml of G418 (Invitrogen, California, USA).
The murine wild type (wt) RV strain EC was obtained from Dr. Harry Greenberg (Department of Medicine and Microbiology and Immunology, Stanford University School of Medicine, Stanford, California). The production and titration of wt RV EC used to challenge mice after vaccination was described previously (6). The simian RV strain SA11 was obtained from Dr. Catherine Eichwald (University of Zurich, Switzerland); it was propagated in MA104 cells and inactivated as previously described (21, 32).

Construction of HSV-1 amplicon vectors

All HSV-1 amplicon vectors used in this study encode enhanced green fluorescent protein (EGFP) to facilitate titration of the amplicon vector stocks and identification of vector-infected cells (15). In addition, the vectors express individual or multiple rotavirus genes and were constructed as follows: (i) $pHSV_D$: The poliovirus IRES element was amplified with primers containing *Cla*I and *Xba*I restriction sites at the 5' ends, respectively (for primer sequences, see Table 1), using plasmid pQuattro (kindly provided by M. Fussenegger, Institute of Biotechnology, Swiss Federal Institute of Technology, ETH Zurich, Switzerland) as template. The resulting PCR product (IRES2) was inserted between the *Cla*I and *Xba*I sites of $pHSV_S$, which contains a transcription unit consisting of the HSV-1 immediate-early (IE) 4/5 promoter, an IRES, and the SV40 polyadenylation signal (15) (ii) $pHSV_T$: A third IRES sequence, derived from encephalomyocarditis virus (EMCV) was amplified with primers containing *Spe*I and *Ecl136*II restriction sites at the 5' ends, respectively, using plasmid pQuattro as the template. The resulting PCR product was inserted between the *Spe*I and *Ecl*136II restriction sites of $pHSV_D$. The resulting HSV-1 amplicon plasmid $pHSV_T$ contains three IRES signals between the HSV-1 IE 4/5 promoter and the SV40 polyadenylation signal.
The HSV-1 amplicon vectors expressing the RV structural genes of strain RRV have been generated as follows (see also Table 1 and Fig 1A): (i) $pHSV_S$[VP2]: The VP2 gene was amplified with primers containing *Sal*I restriction sites at the 5' ends using plasmid pVP2_RRV_Lopez (kindly provided by S. Lopez, Universidad Nacional Autonoma de Mexico (UNAM), Morelos, Mexico) as the template. The resulting PCR product was inserted into the *Sal*I restriction site of $pHSV_S$. (ii) $pHSV_S$[VP6]: The VP6 gene was amplified with primers containing *Sal*I restriction sites at the 5' ends, using plasmid pENTR-VP6_RRV (M. Berois,

Universidad de la Republica, Montevideo, Uruguay) as the template. The resulting PCR product was inserted into the *Sal*I restriction site of $pHSV_S$. (iii) $pHSV_S$[VP7]: The VP7 gene was amplified with primers containing *Asu*II restriction sites at the 5' ends, using plasmid pVP7_RRV_Lopez (S. Lopez, Morelos, Mexico) as template. The resulting PCR product was inserted into the *Asu*II restriction site of $pHSV_S$. (iv) $pHSV_D$[VP6/2]: The VP6 PCR product described under (ii) was also subcloned into the *Sal*I site of $pHSV_D$ resulting in $pHSV_D$[VP6]. VP2 was amplified from plasmid pVP2_RRV_Lopez with primers containing *Asc*I and *Cla*I restriction sites at the 5' ends, respectively, and inserted between the *Asc*I and *Cla*I sites of $pHSV_D$[VP6]. (v) $pHSV_D$[VP7/6]: The VP7 PCR product described under (iii) was inserted also into the *Asu*II site of $pHSV_D$ resulting in $pHSV_D$[VP7]. VP6 was amplified from plasmid pENTR-VP6_RRV with primers containing *Asc*I and *Cla*I restriction sites at the 5' ends, respectively, and inserted between the *Asc*I and *Cla*I sites of $pHSV_D$[VP7]. (vi) $pHSV_T$[VP7/6/2]: The VP7 PCR product described under (iii) was inserted also into the *Asu*II site of $pHSV_T$ resulting in $pHSV_T$[VP7]. The VP6 PCR product described under (v) was also inserted between the *Asc*I and *Cla*I sites of $pHSV_T$[VP7] resulting in $pHSV_T$[VP7/6]. VP2 was amplified from plasmid pVP2_RRV_Lopez with primers containing *Xho*I restriction sites at the 5' ends, and inserted into the *Xho*I site of $pHSV_T$[VP7/6].

Production of HSV-1 amplicon vector stocks

Helper virus-free HSV-1 amplicon vector stocks were prepared as previously described (24, 79, 80). The HSV-1 genome was provided in trans by a bacterial artificial chromosome (BAC) containing the HSV-1 genome with deletions in the DNA cleavage/packaging signals and the essential ICP27 gene (fHSVΔpacΔICP27). Briefly, Vero 2-2 cells were co-transfected with amplicon plasmid DNA, the fHSVΔpacΔICP27 BAC DNA, and plasmid pEBHICP27 (which provides the HSV-1 ICP27 gene in trans), using Lipofectamine and Plus Reagent (Invitrogen, California, USA). After 72 h, cells were scraped into the medium, sonicated, and the cell debris was removed by centrifugation. For titration, Vero 2-2 cells were infected with the amplicon vectors and, after 24 h, green fluorescent cells were counted using an inverted fluorescence microscope (Axio Observer inverted microscope, Zeiss AG, Germany). The titers were determined as transducing units (TU)/ ml and ranged between 2-8 $x10^6$ TU/ml.

Western analysis of vector encoded RV structural proteins

Cells were seeded at a concentration of $1x10^5$ cells/well in 24-well tissue culture plates, and amplicon vectors were added at a multiplicity of infection (MOI) of 0.5 or 1 TU per cell. Total cell lysates were harvested at different time points after infection and separated on 10% SDS-polyacrylamide gels. The fractionated proteins were transferred to nitrocellulose membranes, probed with the primary antibodies, and stained using anti-mouse (Sigma, Missouri, USA) or

anti-rabbit (Southern Biotech, Birmingham, USA) IgG antibodies conjugated with horseradish peroxidase (HRP), followed by detection with Amersham ECL Western Blotting Analysis System (GE Healthcare, UK) according to the manufacturer's instructions. Rabbit anti-rotavirus polyclonal serum raised against whole virus (strain RF, provided by D. Poncet, CNRS/INRA, Gif-sur-Yvette, France), mouse anti-GFP monoclonal antibody (Jl-8, Santa Cruz, California, USA) and mouse anti-actin monoclonal antibody (Sigma, Missouri, USA) were used as primary antibodies. For stripping, membranes were incubated for 15 min with Stripping Buffer (Thermo Scientific, Rockford USA) and washed 3 times with PBS.

Immunofluorescence assays

Vero 2-2 cells were grown on 12 mm coverslips (0.17 mm thick) in 24-well plates and either mock infected or infected with amplicon vectors at a MOI of 0.5 TU per cell. The cells were fixed with 3.7% formaldehyde in PBS and treated with 0.1 M glycine in PBS. After permeabilization with PBS containing 0.2% Triton X-100 (PBS-T), the cells were blocked with PBS supplemented with 3% bovine serum albumin (PBS-BSA). Cells were incubated with rabbit anti-rotavirus serum (strain RF, provided by D. Poncet, Gif-sur-Yvette, France) diluted in PBS-BSA (1:400) for 90 min at RT and then washed three times with PBS. As secondary antibodies, goat anti-rabbit IgG(H+L)-Alexa Fluor 594 or 633 (Molecular Probes, Invitrogen, USA) were used at a dilution of 1:400. Cells were incubated with DAPI (1 µg/ml in PBS, Roche, Switzerland) to visualize nuclei. The ER was stained using lectin ConcanavalinA (ConA) conjugated with Alexa Fluor 594 (20µg/µl in PBS; Molecular Probes, Invitrogen, USA). After washing the cells with PBS and H_2O, the coverslips were mounted in Glycergel (Dako Cytomation, Denmark) containing 25 mg/ml DABCO (Fluka, USA) to retard discoloration. Samples were analyzed using a confocal laser-scanning microscope SP2 (Leica Microsystems, Wetzlar, Germany, 63x oil objective).

Electron microscopy

Cells were seeded at a density of $1.2x10^6$ cells/plate into 6 cm^2 tissue culture plates. The following day, cells were mock infected or infected with HSV-1 amplicon vectors at a MOI of 0.5 or 1 TU per cell. After 48 h, the cells were scraped into the medium and cell membranes were disrupted by repeated cycles of thawing/freezing. The cell debris was removed by centrifugation and filtration through a 0.45 µm filter. The cleared supernatant was loaded onto a 10% sucrose cushion and concentrated at 100,000 g for 2h at 20℃. For protection, protease inhibitor (Protease inhibitor cocktail tablets complete, Mini, EDTA-free, 1 tablet per 10 ml, Roche Diagnostics, Mannheim, Germany) was added to the supernatant. For immune electron microscopy, samples were adsorbed to carbon coated parlodion films mounted on 300 mesh/inch copper grids (EMS, Fort Washington, PA, USA) for 10 min, blocked with PBS

containing 0.1% BSA (PBS-BSA/0.1%) for 10 min, incubated with the polyclonal anti-rotavirus serum at a dilution of 1:1000 PBS-BSA/0.1% for 1 hour, washed several times with PBS-BSA/0.1%, incubated with goat anti rabbit IgG coupled to 12 nm colloidal gold particles (Jackson ImmunoResearch, West Grove, PA, USA), washed several times with PBS and H_20, and stained with 2% phosphotungstic acid, pH 7.0 (Aldrich, Steinheim, Germany) for 1 min. Specimens were analyzed in a transmission electron microscope (CM12, Philips, Eindhoven, The Netherlands) equipped with a CCD camera (Ultrascan 1000, Gatan, Pleasanton, CA, USA) at an acceleration voltage of 100 kV.

RNA isolation and reverse transcription PCR

Cells were seeded at a density of $5x10^5$ cells per well in a six-well plate and, one day later, mock infected or infected with HSV-1 amplicon vectors at an MOI of 1. Total RNA from infected cells was harvested 24 h later using the total RNA purification kit NucleoSpin RNA II (Macherey-Nagel, Germany) according to the instructions provided by the manufacturer. An additional DNase digest was performed by adding 1 µl of DNaseI (Roche, Switzerland) per 50 µl of RNA. The samples were incubated for 15 min at 37℃, inactivated for 10 min at 75℃, and stored at –20℃. Reverse transcription of total RNA was performed using the Reverse Transcription System (Promega) with random primers provided in the kit. As control, the reaction was performed without the enzyme. Per reaction, 1 µg of total RNA was used and incubated for 10 min at RT and for 30 min at 42℃; then the enzyme was inactivated for 5 min at 95℃. The cDNA was used immediately for PCR or stored at –20℃. PCR of the cDNA was performed using the REDTaq ReadyMix PCR Reaction Mix (Sigma, Missouri, USA) and the primers shown in Table 1.

Immunization of mice and sample collection

Five weeks old BALB/c mice were previously confirmed to be negative for anti-rotavirus antibodies by ELISA. Mice were intramuscularly (i.m.) inoculated at days 0 and 21 with: (i) 5×10^5 TU or 1×10^6 TU of HSV_T[VP7/6/2], (ii) 5×10^5 TU or 1×10^6 TU of HSV[EGFP] or (iii) PBS buffer. Individual serum and fecal samples were collected from all mice at 0, 7, 20, 35, and 41 days after the first immunization. All animal procedures were conducted in accordance with the regulations of the Quilmes University Ethic Committee.

Detection of antibody responses by ELISA

For detection of RV specific Igs by ELISA, stool samples (10% in TNC buffer, 10 mM Tris, 140 mM NaCl, 5 mM $CaCl_2$, supplemented with 0.05% Tween 20 and Protease Inhibitor Cocktail; Sigma, Saint Louis, MO) and serum samples were diluted in PBS containing 1% casein and 0.2% Tween 20. Fecal suspensions were diluted 1/5 for IgG or IgA detection, and

serum samples were diluted 1/100 and 1/20 for IgG or IgA detection, respectively. To detect rotavirus specific IgG antibodies, 96-well plates were coated over night at 4℃ with concentrated RV strain RRV in carbonate buffer. Diluted samples were added, and plates were incubated for 1 h at 37℃. HRP-conjugated goat anti-mouse IgG (Fc) antibodies (Pierce Biotechnology, Rockford, IL) were added to the plates for 1 h at 37℃. Between steps, plates were washed three times with 0.2% Tween 20 in PBS. The o-phenylenediamine peroxidase substrate was then used for detection. The reaction was stopped after 15 min with sulfuric acid and the optical density at 490 nm was determined. To detect rotavirus specific IgA antibodies, 96-well plates were coated for 1 h at RT with goat anti-mouse IgA, α chain specific unconjugated antibodies in carbonate buffer. Diluted samples were added, and plates were incubated for 1 h at 37℃. Concentrated rotavirus strain RRV was added and plates were incubated over night at 4℃. After washing, biotinylated goat anti-rotavirus polyclonal IgG was added, and plates were incubated for 1 h at 37℃. HRP-conjugated Streptavidin (United States Biological, Swampscott, MA) was added, and plates were incubated for 30 min at 37℃. Between steps, plates were washed three times with 0.2% Tween 20 in PBS. Detection was performed as described above.

Analysis of antibody responses

Purified and concentrated RV strain RRV proteins were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes. After blocking with PBS containing 1% casein, the membranes were incubated with 1/50 dilutions of serum samples obtained at day 42 after immunization. Mouse hyperimmune serum against RV strain RRV was used as positive control. After washing, membranes were incubated with HRP-conjugated goat anti-mouse IgG (Pierce Biotechnology, Rockford, IL) followed by detection with a chemiluminescent substrate (PBL, Bernal, Argentina) according to the manufacturer's instructions.

Virus challenge and detection of virus shedding

Three weeks after the second immunization, mice were orally challenged with 10^4 shedding doses (SD_{50}) of wt RV strain EC (6). To measure rotavirus shedding, stool pellets were collected from each mouse every day for 8 days after challenge and stored at -80℃. The collected samples were thawed and 10% dilutions were made in TNC buffer containing 0,05% Tween 20 and protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO), and mixed well before debris was removed by centrifugation (2500 g, 10 min). The presence of rotavirus antigen in fecal samples was determined by ELISA as described previously (1). Measurement of protective efficacy of amplicon vectors was based on both the duration and amplitude of virus antigen shedding. Therefore, virus antigen shedding curves (OD versus

days post-challenge) of each animal were plotted and the area under the shedding curve for each animal was calculated and compared to that of the control group (33).

Statistical analysis

Statistical analysis was performed using the program GraphPad Prism (CA, USA). Comparison of the viral shedding and differences between animal groups were compared by Student's t-test. P-Values were considered to be significant if less than 0.05 ($p<0.05$).

Results

Polycistronic HSV-1 amplicon vectors encode structural RV proteins

The HSV-1 amplicon vectors constructed for this study are shown in Fig. 1A. The order of the individual RV genes in the polycistronic vectors was based on the composition of the mature RV particle: the major constituent of the outer layer is glycoprotein VP7, of which 780 copies are grouped as 260 trimers; the intermediate layer is formed by 780 copies of VP6 arranged as 260 trimers; the innermost layer is composed of 120 copies of VP2. We hypothesized therefore that an equimolar ratio of the structural proteins is not strictly required and placed the VP2 gene, which encodes the least abundant structural protein after the second or third IRES. Vector-mediated expression of RV genes VP2, VP6 and VP7 was confirmed by Western analysis of total cell lysates harvested at 24 h after infection (Fig. 1B). As expected, in $pHSV_D$ and $pHSV_T$-infected cells, expression of downstream cistrons was markedly reduced. Accordingly, the intensity of the EGFP band also decreased with increasing position number in the polycistron (Fig. 1B, lower panel).

Next, we examined the synthesis and subcellular localization of the structural RV proteins in HSV-1 amplicon vector-infected cells by indirect immunofluorescence using a polyclonal rabbit anti-RV serum (Fig. 2). EGFP fluorescence was used to identify vector-infected cells. VP2 and VP7 were observed as small foci with some aggregation around the nucleus (Fig. 2A). As previously described (20, 54), the major capsid protein VP6 formed fiber-like structures in the cytoplasm (Fig. 2A). In cells infected with $pHSV_D$[VP6/2], $pHSV_D$[VP7/6], or $pHSV_T$[VP7/6/2], the RV proteins were distributed in a punctuate pattern throughout the cytoplasm and no fiber-like structures were detected (Fig. 2B). As previously observed by Western blot (Fig. 1B), the intensity of EGFP fluorescence decreased with increasing position number in the polycistron (Fig. 2, A and B). In wt RV infected cells, the outer capsid glycoprotein VP7 is a membrane protein located at the endoplasmic reticulum (ER) (20, 59). To determine if VP7 localizes to the ER also when encoded by HSV-1 amplicon vectors, cells were infected with $pHSV_S$[VP7] and, 24 h later, stained with ConA (Fig. 2C) and a polyclonal rabbit anti-RV serum. Confocal laser scanning microscopy revealed that VP7 expressed from HSV-1 amplicon vectors indeed was located at the ER as it co-localized with ConA staining.

Taken together, these results demonstrate that the three RV structural genes VP2, VP6, and VP7 are expressed from the different polycistronic vectors, however with reduced levels when placed after IRES. The subcellular localization of the three vector encoded proteins was comparable to that described for wt RV encoded VP2, VP6, and VP7 proteins (11, 58).

HSV-1 amplicon vectors mediate the generation of rotavirus-like particles (RVLPs) in vector-infected cells

In order to examine the potential assembly of the vector encoded structural proteins into RVLPs, cells were infected with $pHSV_D$[VP6/2] and $pHSV_T$[VP6/2/7] amplicons. After 48 h, cell lysates were prepared for immunogold staining and electron microscopy (Fig. 3). Purified, inactivated wt RV strain SA11 served as positive control, mock-infected cells as negative control (not shown). Infection of cells with $pHSV_D$[VP6/2] resulted in the generation of double-layered particles (Fig. 3A), while infection with $pHSV_T$[VP6/2/7] resulted in the formation of triple-layered RVLPs (Fig. 3B). Lysates from the $pHSV_T$[VP6/2/7] infected cells were also examined by negative staining in absence of immunogold labeling in order to obtain a higher structural resolution. Triple-layered RVLPs were clearly visible (Fig. 3C). Western analysis of the concentrated samples used for electron microscopy confirmed that the observed RVLPs consisted of the RV structural proteins (not shown). The polyclonal antiserum used for immunogold detection efficiently labeled partially disrupted wt RV SA11 particles but not full particles (Fig. 3D). This may be due to the high anti-VP6 activity of the polyclonal antiserum, which can efficiently label the exposed protein in disrupted particles, but not that of complete particles. This could, in part, be due to the serotype specific nature of the exposed epitopes of VP7, which react very poorly with the heterotypic anti-RF antiserum. Taken together, these data demonstrate that the HSV-1 amplicon vectored delivery of structural RV genes supports the assembly of RVLPs in mammalian cells.

Splicing of rotavirus genes expressed from HSV-1 amplicon vectors

As HSV-1 derived vectors deliver transgenes into the host cell nucleus while RV replicates in the cytoplasm, and because several splicing donor and acceptor sites are predicted on the VP6 sequence (Fig. 4A; (91), we examined the VP6 RNA from vector-infected cells for possible splicing events. For this, total RNA was extracted from vector infected or mock infected cells at 24 hpi and reverse transcribed. Subsequent PCR was performed to amplify the complete open reading frame (ORF) of VP6 with a size of approximately 1214 bp (see Fig. 4A). RT-PCR of GAPDH in presence or absence of reverse transcriptase served as control (Fig. 4B, right panel). Interestingly, RT-PCR of cDNA from $pHSV_S$[VP6], $pHSV_D$[VP6/2] or $pHSV_T$[VP7/6/2] infected cells revealed a prominent band of approximately 400 bp in addition to the full-length band (Fig. 4B, left panel). Sequence analysis of the 400 bp band revealed a truncated VP6 gene, in which the middle part of VP6 was deleted, as predicted from the splice donor and acceptor sites. The start and stop codons of the VP6 ORF were still in frame and could potentially give rise to a truncated protein with a calculated molecular weight of approximately 14 kDa. Besides VP6, there are splicing sites predicted also for VP2, but no splicing was detected for either VP2 or VP7 (not shown).

The Vero 2-2 cells used for the experiments described above express the HSV-1 ICP27 gene. ICP27 is an essential immediate early protein, which, besides other functions, can inhibit splicing of both viral and cellular RNA (35, 93). To find out if HSV-1 amplicon vector-mediated production of rotavirus proteins and RVLPs is possible only in cells in which splicing is inhibited, we next analyzed protein synthesis and RVLP production in vector infected, parental Vero cells. No differences concerning RV protein synthesis (Fig. 4C) or RVLP structure (Fig. 4D) were observed between Vero and Vero 2-2 cells infected with HSV_S[VP6] (Fig. 4C) or $pHSV_D$[VP6/2] (Fig. 4D). The intensity of the band corresponding to full length VP6 protein was comparable between Vero and Vero 2-2 cells, indicating that even if splicing of VP6 RNA occurred, there was no major decrease in the production of the full length protein. Moreover, the use of splicing inhibitors did not result in increased accumulation of vector encoded VP6 protein (not shown).

Immunization of mice with $pHSV_T$[VP7/6/2] resulted in partial protection from virus challenge

After demonstrating the assembly of the vector-encoded RV proteins into RVLPs, the potential usefulness of these HSV-1 amplicon vectors for vaccination against RV infection was evaluated by assessing their ability to induce specific antibody responses in vector-vaccinated mice. For this, Balb/c mice were inoculated i.m. with HSV_T[VP7/6/2] or, as control, with the empty vector pHSV[EGFP] or PBS in a prime-boost regimen using a dose of 5×10^5 or 1×10^6 TU per inoculation (Fig 5A). RV-specific antibodies in serum and feces collected from mice at days 0, 7, 20, 35, and 41 after the first immunization were determined by ELISA and Western analysis as described in Material and Methods. Significant differences in the average level of serum or fecal IgG and IgA antibody levels between mice immunized with HSV_T[VP7/6/2] and the control mice immunized with pHSV[EGFP] could not be observed by ELISAs (data not shown). However, after the second immunization VP6 specific antibodies were detected in all serum samples by Western analysis (Fig. 5B). VP2 specific antibodies were observed only in three out of seven sera (Fig. 5B, lanes 3, 4, 7), while VP7 specific antibodies were not detected in any of the samples analyzed. Series of experiments performed with doses of either 5×10^5 (Fig. 5B, lanes 1-4) or 1×10^6 TU (Fig. 5B, lanes 5-7) per inoculation yielded similar results. RV-specific fecal or serum IgA antibodies and fecal IgG antibodies of mice immunized with HSV_T[VP7/6/2] were below the sensitivity of the ELISA.

Next, we addressed the question whether vector immunized mice are protected from oral challenge with wt RV. In the adult mouse model, infection with RV does not induce disease and protection is therefore defined as the absence of detectable fecal viral antigen following challenge, and partial protection is defined as reduced quantities of fecal viral antigen compared to that shed by PBS-inoculated mice after challenge (10, 100). Accordingly,

protection from RV infection upon challenge was evaluated by comparing antigen shedding in mice vaccinated i.m. with two doses of $pHSV_T$[VP7/6/2] or pHSV[EGFP] to mock vaccinated mice (PBS). Animals were orally challenged three weeks after the second immunization with live RV strain EC (10^4 SD_{50}). Fecal samples were collected from day 0 to 8 post challenge, and antigen levels were monitored by ELISA (Fig. 5C). Protection against infection was determined as total viral shedding in feces by calculating the area under the curve (AUC). The percent reduction in shedding was determined by comparing the AUC of the vaccinated mice to the mean AUC of the PBS control group. Mice immunized with two doses of 1 x 10^6 TU of $pHSV_T$[VP7/6/2] were partially protected from RV infection. They demonstrated a significant decrease ($p<0.05$) in the shedding of RV (39.9% reduction) in feces after oral challenge with wt RV EC compared to control mice (Fig. 5C). Mice immunized with 5 x 10^5 TU of $pHSV_T$[VP7/6/2] had a non-significant level of reduction of RV antigen shedding in feces (18.7%, $p>0.05$). Taken together, immunization of mice with $pHSV_T$[VP7/6/2] resulted in a dose dependent partial protection of vaccinated mice as defined for the adult mouse model. However, only a low antibody response was detectable, which suggests that this protection did not correlate with the variable antibody response observed.

Discussion

The use of VLPs as vaccines is very promising because of their safety compared to live attenuated virus vaccines. Despite the lack of any genetic material, VLPs retain the high immunogenicity of the parental viral particle. VLPs could serve also as carriers of foreign epitopes from bacterial or viral pathogens or drugs, which could enhance the immune response to the applied vaccine. Moreover, VLPs containing structural proteins from different serotypes could be combined. The use of a mammalian delivery system, such as HSV-1 amplicon vectors to launch the in situ production of heterologous VLPs, provides the advantage that time consuming and expensive purification of VLPs is not required. Indeed, here we demonstrated that HSV-1 amplicon vectors can mediate the efficient in situ production of RV structural proteins and RVLPs in vector-infected cells, despite the facts that (i) the normal site for RV replication locates in so-called viroplasms the cytoplasm while HSV-1 amplicon vectors deliver the RV genes into the nucleus, (ii) and several splicing sites are predicted in the ORF of VP6 and VP2 (91). However, our results show that splicing of VP6 RNA did not prevent the production of the full-length protein and the use of splicing inhibitors did not result in increased production of vector-encoded VP6 protein or RVLPs. When used as vaccine vectors in the adult mouse model (100), a RV VP6 specific antibody response was detected in the sera of all immunized animals. Although RV-specific fecal or serum IgA antibodies or fecal IgG antibodies were below the detection limit, significant partial protection from challenge with wt RV was observed in the immunized mice. The molecular mechanisms regulating protection against RV are still unclear and the immunological mediators remain to be established. In the mouse model, different immune effectors appear to be responsible for protection depending on whether immunity is elicited by natural infection, vaccination with attenuated strains, or immunization with non-living vaccines (3). Also the protective mechanisms are different depending on the inoculation route, adjuvant and even on the source and level of purification of the preparation (46, 75). Both in humans and animal models, protection after natural infection or oral vaccination with live attenuated RV moderately correlates with specific serum and fecal immunoglobulins, particularly intestinal dimeric IgA (25, 45), indicating that neutralizing antibodies can play a role in protection. However, in some animal models, and particularly through the extensive use of the adult mouse model to address mechanistic questions, alternative ways of protection were clearly demonstrated (12, 23, 104). While protection induced by subunit vaccines is dependent exclusively on CD4+ T cells (64), the same does not apply when attenuated RV is used for vaccination. In this case, the protection does not solely rely on IgA (70) or T cells (26).

Infection with wild-type HSV-1 results in a potent host immune response involving both, the innate and the adaptive immune system (49), but the virus is also able to evade host

immunity through a variety of mechanisms based on immuno-modulatory gene products such as the virion host shut-off protein (81) or the ICP47 protein (106). Although HSV-1 amplicon vectors do not express any viral genes, including immuno-evasive genes, they are composed of some 40 different virus-encoded structural proteins, which are delivered into the cell during infection. This could lead to intrinsic cellular responses, triggering tissue alarm signals and innate responses like cytokine secretion, and promoting a bias to a TH1-like adaptive immune response. Hence, as HSV-1 amplicon vectors trigger per se a TH1 response, further adjuvants are not necessary.

Due to the high prevalence of HSV-1 in humans, one major concern about the use of HSV-1 derived vectors as vaccines is the possible impact of pre-existing antiviral immunity, which may lead to vector neutralization or rapid clearance of vector-transduced cells. This issue was addressed in one study where mice were immunized with a heterologous HSV-1 strain to generate an anti-HSV-1 immune response and then subsequently inoculated with helper virus-free HSV-1 amplicons vectors. Although significant titers of neutralizing antibodies were present in those mice, HSV-1 amplicon vector mediated cellular immune responses were only modestly reduced (36), indicating that pre-existing immunity may not prevent the successful use of HSV-1 amplicon vector-based vaccines.

HSV-1 amplicon vectors have been used to express HIV envelope glycoprotein gp120 (36). A single dose of 1 x 10^6 transducing units of gp120 expressing HSV-1 amplicon vectors resulted in strong antigen-specific and long-lasting cellular and humoral responses in mice. Subsequent work showed that amplicon vector-induced immune responses could be strongly enhanced by prior priming with either a DNA vaccine encoding the same antigen or by initial priming with the amplicon vectors (99). Taken together, amplicon vectors have shown promising results as vaccine delivery system in several preclinical studies, including animal models for cancer treatment (56, 78, 86, 94) and prophylactic immunizations against infectious pathogens (31, 36, 53, 84). HSV-1 amplicon vectors have also been used to express the foot and mouth disease virus (FMDV) structural genes resulting in the assembly of FMDV empty particles. Mice vaccinated in a prime/boost regimen with 5 x 10^5 transducing units of this amplicon vector elicited a FMDV-specific humoral response in the absence of adjuvants and were partially protected against challenge with a high dose of live FMDV (15).

The present work suggests that HSV-1 amplicon vectors are able to elicit antigen-specific immune responses to the RV proteins and, at least, partial protection against challenge with wt RV. Perhaps these responses could be further enhanced by evaluating different routes of vaccination. HSV-1 amplicon vectors have many properties that make them promising vaccine vectors. Their large transgene capacity (up to 152 kbp) allows the simultaneous encapsidation of multiple copies of a transgene/antigen or the expression of multiple different transgenes/antigens, as shown in this work. In particular, amplicon vectors are safe because

they can be produced in the absence of helper virus and, therefore, do not express any HSV-1 genes (24, 71, 79, 80). The production of helper virus-free amplicon vector stocks is based on co-transfection of amplicon plasmids with HSV-1 helper genomes and is therefore limited as the vectors cannot be further amplified. Further optimization and refinement of the currently used helper virus-free systems, like a more efficient delivery of the BAC helper genome into mammalian cells, will be required to achieve titers high enough for clinical testing. At present, no method combining a total absence of contaminating helper particles with ability to produce very large amounts of amplicon particles is available. However, other packaging systems have been developed which allow the production of safe and high titered amplicon vector stocks that contain 0.05 to 1.0 % of defective, non-pathogenic helper viruses (14, 57, 107). In addition to improvements to vector production, the amplicon vector itself could be further optimized for use as a vaccine vector. For example, immuno-modulating genes like the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) could be included in the vectors to co-stimulate the immune cells and to enhance the humoral and cellular immune response (19, 92, 103). For RNA virus genes, sequences should be adapted for the expression in mammalian cells; cryptic introns modified and splice donor/acceptor sites mutated. The polycistronic amplicon vector used here resulted in a reduced level of expressed transgene from the downstream cistrons (Fig. 1B, 2). The use of individual promoters for the different transgenes or the replacement of one or two IRES by the viral cleavage factor 2A of FMDV, a feature widely used in bicistronic vectors (8, 40), could be another strategy to enhance transgene expression and amount of RVLPs produced.

In conclusion, the presented work suggests that HSV-1 amplicon vectors are able to elicit antigen-specific immune responses to the RV proteins and partial protection against challenge with wt RV, opening the possibility of using the described vectors in prophylactic vaccinations against RV infections.

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FIGURE LEGENDS

Figure 1. RV genes expressed from HSV-1 amplicon vectors.

A) Schematic representation of the HSV-1 amplicon vectors expressing or co-expressing RV genes VP2, VP6 and VP7. Polycistronic expression is facilitated by two picornavirus IRES and an encephalomyocarditis virus (EMCV) derived IRES (28), and controlled by the HSV-1 IE4/5 promoter. All vectors contain the EGFP reporter gene to support titration of vector stocks. The HSV-1 origin of DNA replication (ori_S) and packaging/cleavage signal (pac) as well as the restriction sites used for cloning are indicated. B) Vero 2-2 cells were infected with HSV-1 amplicon vectors (MOI 1), and total cell lysates were harvested at 24 hpi. Transgene expression was analyzed by Western blot using a polyclonal rabbit anti-RV serum for detection of RV proteins or a monoclonal anti-GFP antibody to stain EGFP. Detection of actin was used as loading control. Purified and inactivated wt RV strain SA11 served as positive control.

Figure 2. Intracellular distribution of HSV-1 amplicon vector encoded RV proteins.

Vero 2-2 cells were infected with HSV-1 amplicon vectors (MOI 0.5). The cells were fixed 24 hpi, permeabilized and stained with a polyclonal anti-RV serum (anti-RV) and an Alexa Fluor594 conjugated secondary antibody (red) and analyzed using an inverse confocal laser-scanning microscope. EGFP fluorescence served to identify vector-infected cells (green); nuclei were stained with DAPI (blue). Scale bars = 5 µm. A) Cells infected with HSV_S encoding VP2, VP6 or VP7. B) Cells infected with HSV_D and HSV_T encoding different combinations of VP2, VP6 and VP7. C) HSV-1 amplicon vector encoded VP7 is localized at the ER, as determined by staining with Alexa Fluor594-conjugated lectin ConA (purple).

Figure 3. Electronmicrographs of HSV-1 amplicon vector encoded RVLPs.

Vero 2-2 cells were infected with HSV-1 amplicon vectors (MOI 1). Two days post infection, RVLPs were purified over a sucrose cushion and the concentrated particles were analyzed by electron microscopy. (A, B, D) Immunogold staining using a polyclonal anti-RV serum and a secondary antibody coupled to 12 nm gold particles. (A) Double-layered RVLPs from cells infected with HSV_D[VP6/2]. (B) RVLPs from cells infected with HSV_T[VP7/6/2]. (C) Negative staining of RVLPs from cells infected with HSV_T[VP76/2]. (D) Purified inactivated wt RV strain SA11. Scale bars = 50 nm.

Figure 4. The HSV-1 amplicon vector encoded VP6 RNA is spliced.

A) Schematic representation of the predicted splicing donor and acceptor sites on the VP6 RNA. Primers used for detection of the VP6 ORF are indicated as VP6 fwd and VP6 rev

(Table 1). The amplified full length VP6 ORF (1214 nt) and the truncated form of VP6 (387 nt) are shown. B) Reverse transcription PCR of total RNA isolated from infected cells (see Table 1 for primer sequences). Left panel: Amplification of VP6 sequences using the primers VP6 fwd and VP6 rev. The detected bands correspond to the sizes predicted for full length and truncated VP6 sequences. Right panel: Amplification of GAPDH sequences was used as control. Reactions were performed in presence (+) or absence (-) of reverse transcriptase. Negative control: mock-infected cells. C) Immunoblot of HSV_S[VP6] infected Vero and Vero 2-2 cells. The polyclonal anti-RV serum was used for detection of RV proteins, a monoclonal anti-GFP antibody to stain EGFP, and actin staining as loading control. Purified and inactivated wt RV strain SA11 served as positive control. D) Electronmicrograph (negative staining) of double-layered RVLPs produced in Vero cells infected with HSV_D[VP6/2]. RVLPs purified from total cell lysates were concentrated over a sucrose cushion. Scale bar = 100 nm.

Figure 5. Immunization of mice with HSV_T[VP7/6/2] confers partial protection from challenge with wt RV.

A) Timetable indicating the immunization schedule. Mice (four to six mice per group) were immunized (i.m.) at day 0 and boosted at day 21 with 5 x 10^5 TU or 1 x 10^6 TU of HSV_T[VP7/6/2] or HSV[EGFP]. A third group of mice received PBS injections at days 0 and 21. Stool and serum samples were collected at days 0, 7, 21, 35 and 42 after the first immunization. Three weeks after the second immunization, mice were orally challenged with 10^4 SD_{50} of wt RV strain EC, and stool samples were collected every day for eight days post challenge. B) Western analysis of RV specific antibodies in sera of immunized mice. RV (strain RRV) proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes individually probed with sera diluted 1/100 from mice immunized with 5 x 10^5 TU (lanes 1 to 4) or 1 x 10^6 TU (lanes 5 to 7) of HSV_T[VP7/6/2] or HSV[EGFP] (lanes 8 and 9) are shown. Mouse hyperimmune serum against RV strain RRV was used as positive control to identify VP2, VP6 and VP7. The results shown represent one of two independent experiments. C) Mice immunized with HSV-1 amplicon vectors were challenged as shown in A). The presence of RV antigen in stool was measured by ELISA and virus antigen shedding curves of each animal were plotted, and the area under the shedding curve (AUC) for each animal over 8 days was calculated and compared to that of the control group (PBS, 100 %). Mean AUC per group are shown. Error bars indicate the standard deviation of the mean AUC per group. Differences between groups were compared by Student's t-test. Asterisk: statistically significant ($p<0,05$).

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Figure 1

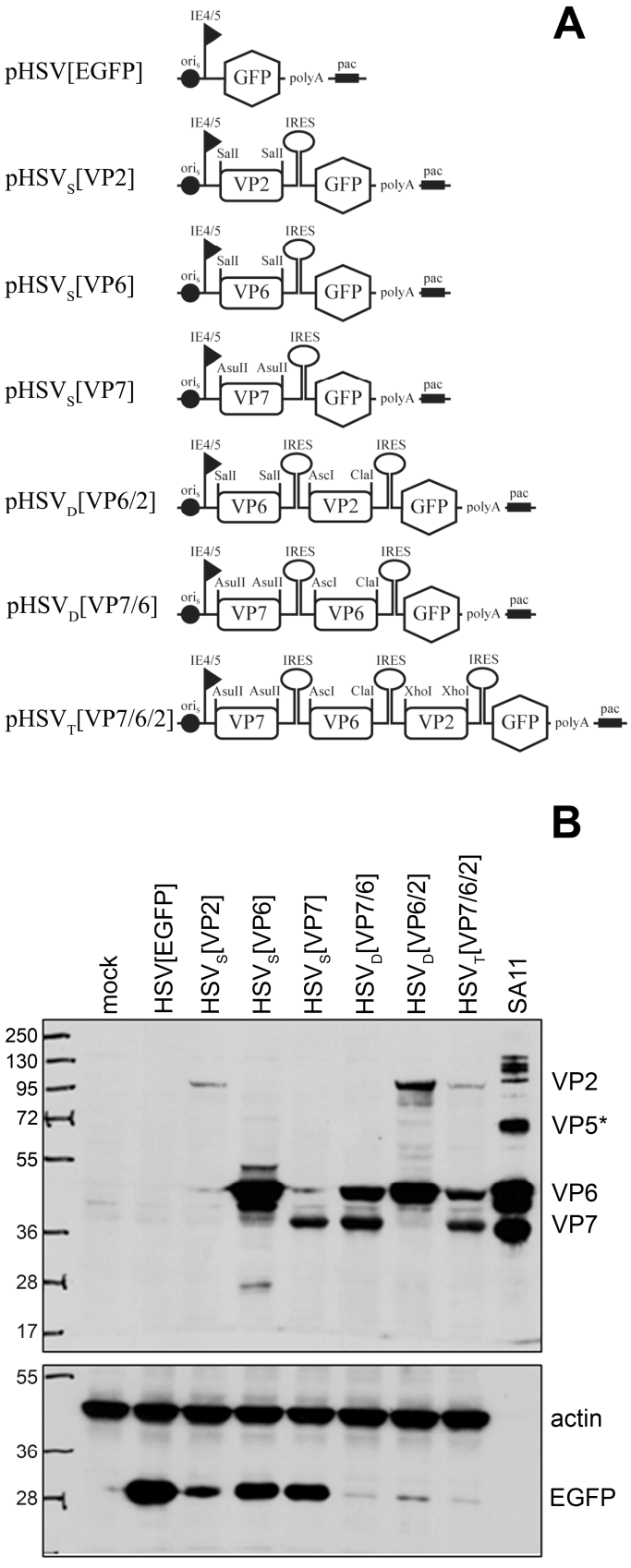
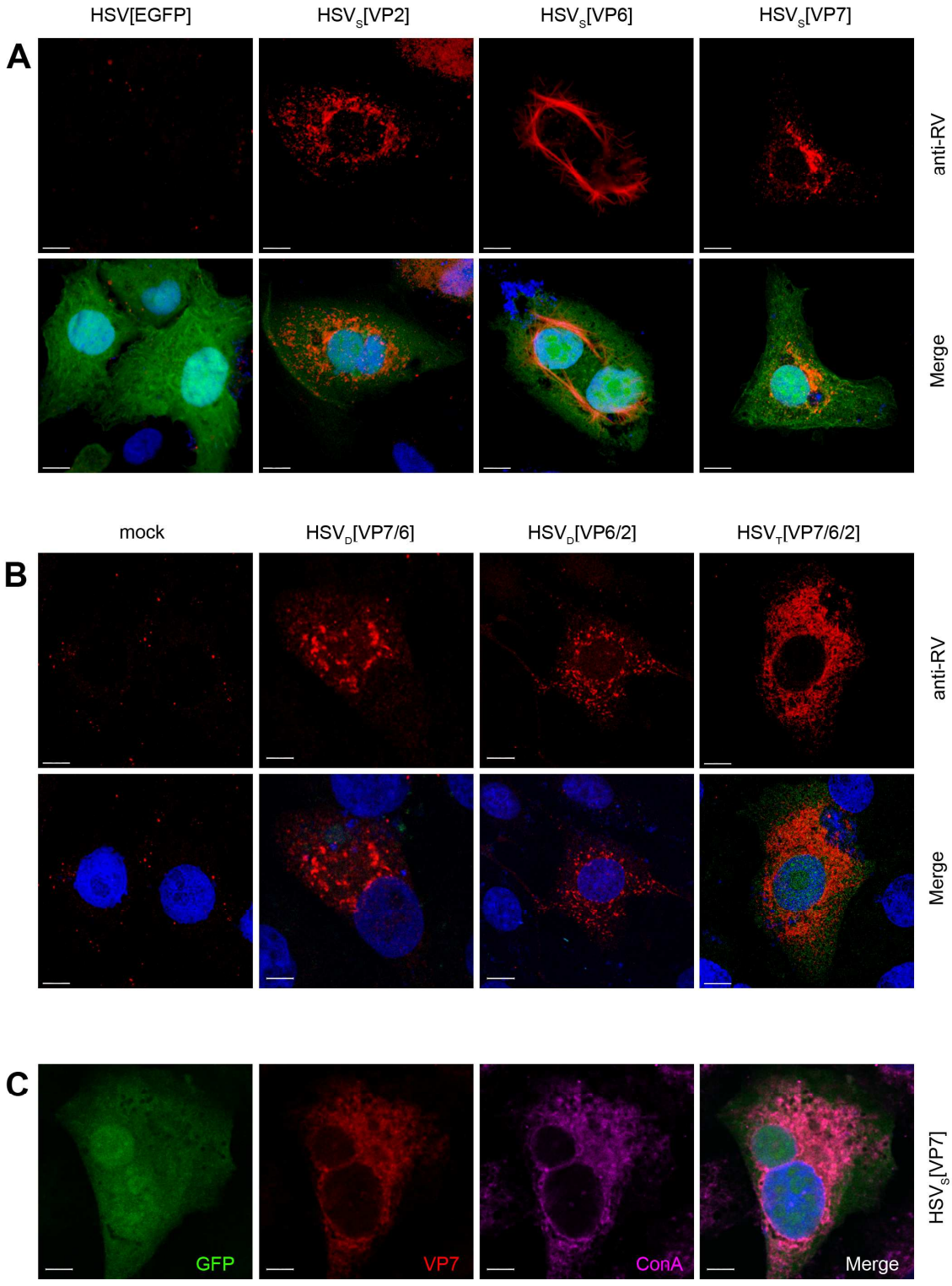

A
pHSV[EGFP]
pHSV$_S$[VP2]
pHSV$_S$[VP6]
pHSV$_S$[VP7]
pHSV$_D$[VP6/2]
pHSV$_D$[VP7/6]
pHSV$_T$[VP7/6/2]
IE4/5
IRES
ori$_S$
SalI
AsuII
AscI
ClaI
XhoI
VP2
VP6
VP7
GFP
polyA
pac
B
mock
HSV[EGFP]
HSV$_S$[VP2]
HSV$_S$[VP6]
HSV$_S$[VP7]
HSV$_D$[VP7/6]
HSV$_D$[VP6/2]
HSV$_T$[VP7/6/2]
SA11
250
130
95
72
55
36
28
17
VP2
VP5*
VP6
VP7
55
36
28
actin
EGFP

Figure 2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HSV[EGFP]
HSV$_S$[VP2]
HSV$_S$[VP6]
HSV$_S$[VP7]
anti-RV
Merge
B
mock
HSV$_D$[VP7/6]
HSV$_D$[VP6/2]
HSV$_T$[VP7/6/2]
anti-RV
Merge
C
GFP
VP7
ConA
Merge
HSV$_S$[VP7]

Figure 3

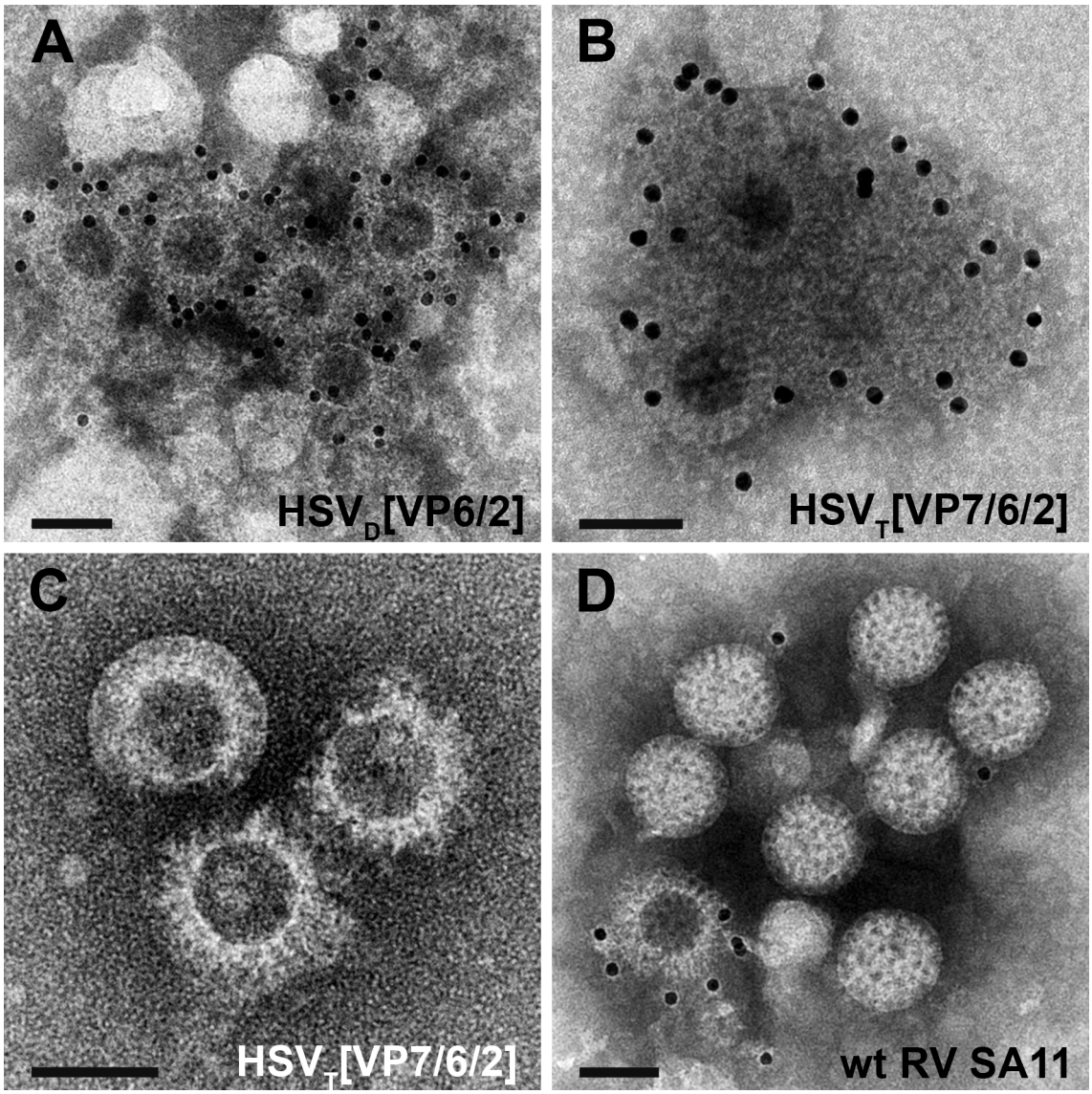

Figure 4

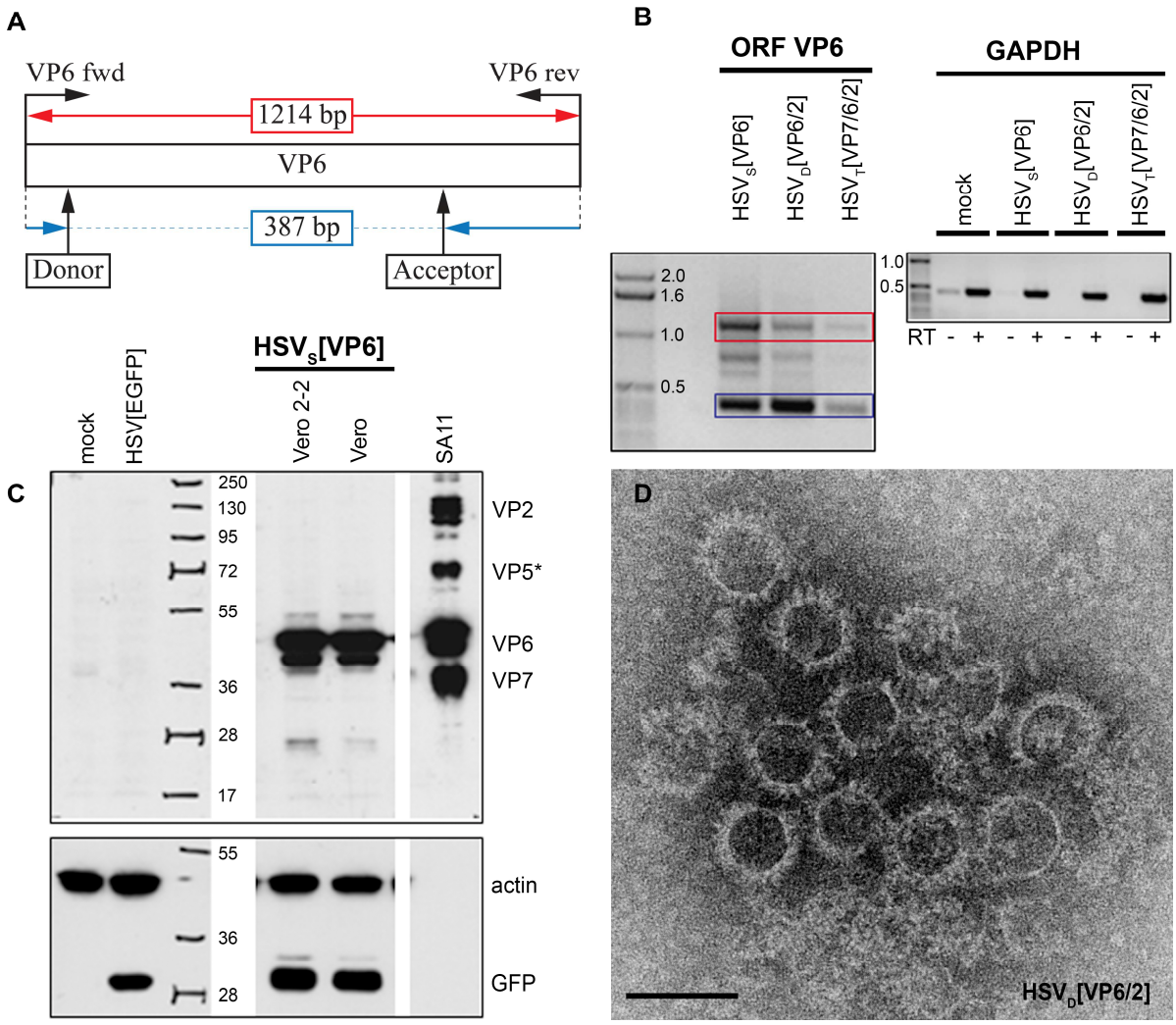
A
VP6 fwd
VP6 rev
1214 bp
VP6
387 bp
Donor
Acceptor
B
ORF VP6
GAPDH
HSV$_S$[VP6]
HSV$_D$[VP6/2]
HSV$_T$[VP7/6/2]
mock
2.0
1.6
1.0
0.5
RT - + - + - + - +
C
HSV$_S$[VP6]
mock
HSV[EGFP]
Vero 2-2
Vero
SA11
250
130
95
72
55
36
28
17
VP2
VP5*
VP6
VP7
actin
GFP
D
HSV$_D$[VP6/2]

Figure 5

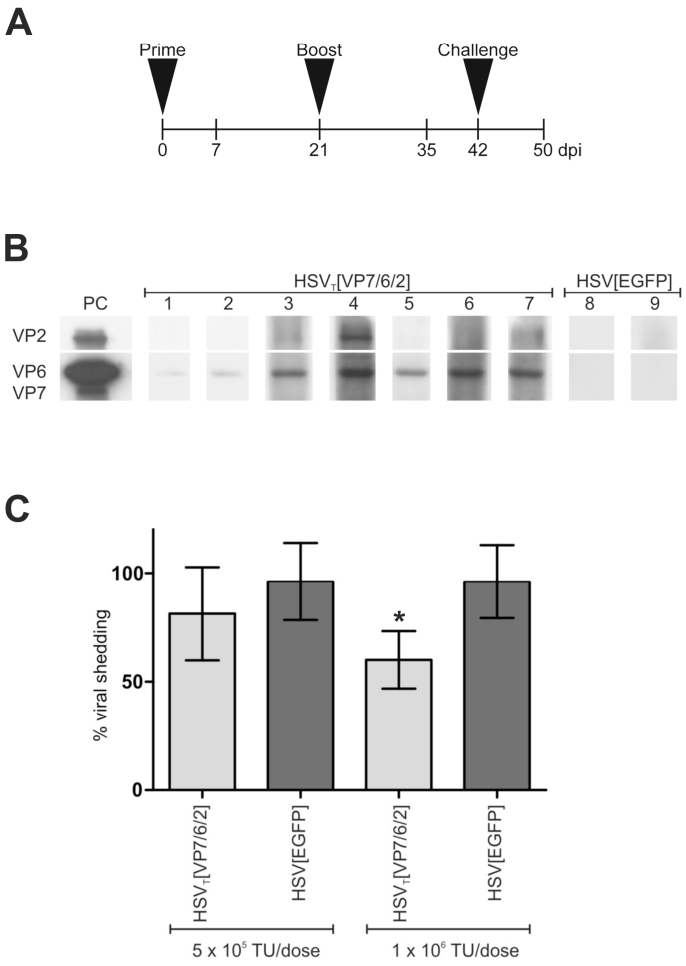
A
Prime
Boost
Challenge
0
7
21
35
42
50 dpi
B
PC
HSV1[VP7/6/2]
HSV[EGFP]
1
2
3
4
5
6
7
8
9
VP2
VP6
VP7
C
% viral shedding
100
50
0
HSV1[VP7/6/2]
HSV[EGFP]
HSV1[VP7/6/2]
HSV[EGFP]
5 x 10^5 TU/dose
1 x 10^6 TU/dose
*

Table 1 Primer list

Gene	Primer fwd	Primer rev	Template	Construct(s)
IRES2	gcctgatcaATCGATgtttaaactta	aattTCTAGAtcgcactagtctcgagaatcca	pQuattro3[1]	$pHSV_D$
CITE	ctttACTAGTacaggtgtccactcccaggtcca	ccatGAGCTCtgcaggaattatcccggggttgt	pQuattro3[1]	$pHSV_T$
VP2	ttaaGTCGACatggcgtacagaaagcgtggag	tagtGTCGACttacagttcgttcatgatgcg	S.Lopez	$pHSV_S$[VP2]
VP2	aatGGCGCGCCatggcgtacagaaagcgtggag	tatATCGATttacagttcgttcatgatgcg	S.Lopez	$pHSV_D$[VP6/2]
VP2	ttaaCTCGAGatggcgtacagaaagcgtggag	tagtCTCGAGttacagttcgttcatgatgcg	S.Lopez	$pHSV_T$[VP7/6/2]
VP6	aggaGTCGACatggatgtcctatactctttg	agctGTCGACtcatttaatgagcatgcttct	pENTR-VP6_RRV[2]	$pHSV_S$[VP6] $pHSV_D$[VP6/2]
VP6	gcagGGCGCGCCatggatgtcctgtactccttg	agctATCGATtcatttgacaagcatgcttct	pENTR-VP6_RRV[2]	$pHSV_D$[VP7/6] $pHSV_T$[VP7/6/2]
VP7	ctctTTCGAAatggactttattatttacaga	ggtgTTCGAActatattctataatagaatgca	S.Lopez	$pHSV_S$[VP7] $pHSV_D$[VP7/6] $pHSV_T$[VP7/6/2]
EGFP	agccgctaccccgaccacat	ttgctcagggcggactgggt	cDNA	RT-PCR
GAPDH	ggggagccaaaagggtcatcatct	acgcctgcttcaccaccttcttga	cDNA	RT-PCR

1 M. Fussenegger, ETHZ, Zurich, Switzerland
2 M. Berois, Montevideo, Uruguay

2.2 Results Part II: Foot-and-Mouth disease virus

2.2.1 Aim of the project

FMDV is the type species of the genus *Aphtovirus* of the *Picornaviridae* family. The highly infectious disease caused by this virus continues to be of major economic importance across the world. The FMDV genome consists of a single copy of a positive-sense, single-stranded RNA, which encodes a single open reading frame. This open reading frame gives rise to a polyprotein, which is proteolytically processed by virus-encoded proteases. The 3C protease cleaves the capsid precursor protein to produce all the capsid proteins. FMDV has a small non-enveloped icosahedral capsid with a diameter of around 25 nm. The capsid is formed from 60 copies each of the four viral structural proteins VP1, VP2, VP3 and VP4. Natural FMDV empty particles have been shown to stimulate the same level of neutralizing antibodies as full particles.

The aim of this project was to construct HSV-1 amplicon vectors, which co-express the FMDV structural proteins and the 2A and 3C proteases; and to evaluate the capability of the vectors to support the *in situ* production of FMDV-like particles (VLPs) in the vector-transduced cells. The potential use of these vectors for the generation of genetic vaccines was assessed by vaccination of mice and evaluation of specific immune responses to the corresponding antigens.

This project was designed and performed in collaboration with the laboratory of CEVAN-CONICET, Buenos Aires, Argentina.

2.2.2 Paper FMDV

HSV-1 amplicon vectors that direct the in situ production of foot-and-mouth disease virus antigens in mammalian cells can be used for genetic immunization

Manuscript published in Vaccine 28 (2010)

Own contribution to the manuscript

Cloning of the HSV-1 amplicon vectors

Characterization of the gene expression by immune fluorescence

Production of vector stocks for immunization of mice

Analysis of VLPs by electron microscopy (assistance from E. Schraner)

Inoculation of mice for immune histochemistry

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HSV-1 amplicon vectors that direct the in situ production of foot-and-mouth disease virus antigens in mammalian cells can be used for genetic immunization

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ABSTRACT

HSV-1 amplicon vectors encoding heterologous antigens were capable to mediate in situ generation of protein synthesis and to generate a specific immune response to the corresponding antigens. In this study, foot-and-mouth disease (FMD) virus antigens were used to generate a genetic vaccine prototype. The amplicons were designed to provide a high safety profile as they do not express any HSV-1 genes when packaged using a helper virus-free system, and they are able to encapsidate several copies of the transgene or allow the simultaneous expression of different genes. Virus-like particles were produced after cell processing of the delivered DNA. Inoculation of mice with 5×10^5 transducing units of amplicon vectors resulted in FMDV-specific humoral responses in the absence of adjuvants, which were dependent on the in situ ***de novo*** production of the vector-encoded antigens. Challenge of mice vaccinated with these amplicons with a high dose of live virus, resulted in partial protection, with a significant reduction of viremia. This work highlights the potential use of a HSV-1 amplicon vector platform for generation of safe genetic vaccines.

1. Introduction

Herpes simplex virus type 1 (HSV-1) based vectors have been evaluated for gene therapy [1–3] and are considered promising vehicles to deliver antigens and immune-modulators for prophylactic and therapeutic vaccination, as well as for fundamental research. Specifically, HSV-1 amplicon vectors have a large transgene capacity (up to 150 kb), which allows the encapsidation of multiple genes or multiple copies of a transgene. Additional advantages of HSV-1 derived vectors include (i) low toxicity and low immunogenicity, in particular when helper virus-free amplicon vectors are used, (ii) high transduction efficiencies in dividing and non-dividing cells from most mammalian species, including antigen-presenting cells in vivo, (iii) genetic stability, and (iv) strong adjuvant effects, very long-lived immune responses, and the capacity of generating both humoral and cellular immune response and mucosal immunity [4].

The use of HSV-1 amplicons for clinical vaccination purposes has not been reported. In this work, this system was used to deliver foot-and-mouth disease (FMD) virus capsid proteins to cells and mice. FMDV is a non-enveloped icosahedral virus of 26 nm in diameter that belongs to the Aphthovirus genus from the Picornaviridae family. The genome is a positive single-stranded RNA of approximately 8.4 kb that encodes for a large polyprotein, which is processed by virus-encoded proteases to produce functional structural and nonstructural proteins. Processing of P12A polypeptide is effected by the 3C protease to produce the capsid proteins VP0, VP3 and VP1 [5]. Cleavage of the VP0 polypeptide into VP2 and VP4 occurs late during virion morphogenesis, upon encapsidation of genomic RNA into provirions [6]. The icosahedral FMDV mature particle consists of 60 copies of each of the four capsid proteins VP1, VP2, VP3, and VP4 [6–8].

Different particulated intermediates have been identified in infected cell extracts and from in vitro translation experiments, which can be separated in density gradients, where intact virions run as 140S. These viral subparticles are: (i) the 5S protomer, which contains a single copy of VP0, VP1 and VP3, (ii) the 12S pentamer, consisting of five copies of the 5S protomer, and (iii) the 75S empty capsids, containing 12 copies of the 12S pentamer, devoid of genomic RNA [6,9]. Natural FMDV empty particles have been shown

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to bind to susceptible cells [10] and to maintain the continuous and discontinuous B-cell epitopes present in authentic virions [11].

Systematic vaccination with chemically inactivated virus particles has been very effective to control FMD in the countries where the disease is enzootic and in FMD-free-with-vaccination countries or zones [12]. A number of concerns and limitations with the use of conventional FMD vaccines remain, and include production in high-containment bio-security facilities, serotype-dependence, and difficulties to distinguish vaccinated from infected or convalescent animals.

Subunit vaccines based on highly immunogenic FMDV proteins or peptides, as well as DNA vaccines, have been studied extensively (reviewed in Ref. [13]). Empty capsids of FMDV have been obtained by recombinant DNA technology in *Escherichia coli*, recombinant baculovirus or vaccinia virus infected cells [9,14,15]. Recombinant capsids have several advantages as vaccine antigens, such as safety, authentic antigenicity, and negative markers (absence of 3D polymerase and other nonstructural proteins). Alternatively, viral vectors capable of expressing FMDV capsids in the target animals have been used, such as live replication-defective human adenovirus vectors [16,17].

In this study, genomic regions of FMDV have been used as a model for testing genetic vaccines based on HSV-1 amplicons.

2. Materials and methods

2.1. Cells and viruses

African green monkey kidney 2-2 cells (Vero2-2) [18] and human embryonic kidney cells (HEK293) were maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin G, 100 μg/ml of streptomycin, 0.25 μg/ml of amphotericin B, and 500 μg/ml of G418 (Invitrogen, CA, USA).

2.2. RT-PCR amplification and cloning of FMDV genomic regions

Genomic RNA from FMDV strain O1/Campos/Brazil/58 (O1/Campos) was provided by the Argentine Animal Health Service (SENASA). Reverse transcription (RT) and PCR amplification was performed on total RNA using the Access RT-PCR kit (Promega, Madison, USA) following the manufacturer's instructions. For the construction of the P12A3C cassette, the P12A fragment was inserted at the *Eco*RV site of plasmid pBBR1MCS2 (pBBR[P12A]). The 3C fragment was inserted between the *Hind*III and *Stu*I restriction sites of pBBR[P12A]. This final construct was designated pBBR[P12A3C] and contained the entire P12A3C expression cassette, flanked by an ATG codon upstream of the P1 coding region and a TAG termination codon located immediately downstream of the 3C coding region.

2.3. Construction of HSV-FMDV amplicon vectors

An HSV-1 amplicon vector expressing the enhanced green fluorescent protein (EGFP) reporter gene and FMDV genomic regions was generated as follows:

(i) *pHSV-EGFP*: The ORF for the EGFP gene was amplified and inserted between the *Sac*I and *Eco*RI sites of the HSV-1 amplicon plasmid pHSVPrPUC (kindly provided by H. J. Federoff, School of Medicine and Dentistry, University of Rochester, Rochester, NY).

(ii) *pHSV$_S$*: The DNA fragment encoding the internal ribosomal entry site (IRES) element of poliovirus was PCR amplified using plasmid pQuattro3 as the template (kindly provided by M. Fussenegger, Swiss Federal Institute of Technology, Zurich [19]) and inserted between the *Sal*I and *Xba*I sites of plasmid pHSV-EGFP.

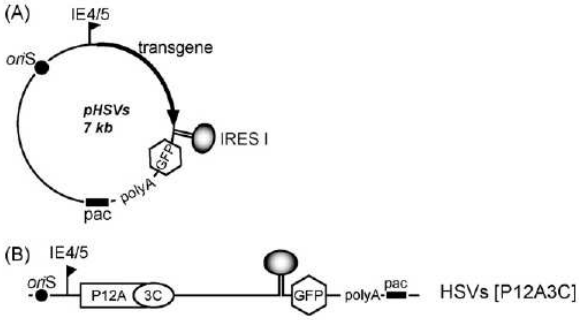

Fig. 1. Schematic representation of HSV-1 derived amplicon plasmid. (A) General features of the novel HSV amplicon plasmid pHSV$_S$ and (B) Schematic representation of the HSV-1 derived amplicon carrying FMDV transgenes. P12A: FMDV capsid proteins precursor plus 2A protein; 3C: FMDV 3C protease; pac: herpesvirus packaging signal; oriS: HSV-1 origin of DNA replication. IE4/5: immediately early herpesvirus promoter. IRES I: picornaviral IRES.

Plasmid pBBR[P12A3C] was used as template for PCR amplification of the whole P12A3C cassette, in order to generate the amplicon plasmid pHSV$_S$[P12A3C] (Fig. 1).

The same FMDV DNA region was inserted in an adenovirus (Ad) vector, Ad[P12A3C], using the commercial kit ViraPower Adenoviral Expression System (Invitrogen, CA, USA), following to the manufacturer's instructions. The Ad vector was propagated and titrated in HEK293A cells, which provide in *trans* the essential E1 protein.

2.4. Packaging of amplicon DNA into HSV-1 virions and titration of vector stocks

Helper virus-free stocks of HSV$_S$[P12A3C] amplicon vector were prepared as previously described [3,20,21]. The HSV-1 genome was provided in *trans* by a bacterial artificial chromosome (BAC) deleted in the essential ICP27 gene (fHSVΔpacΔICP27). Briefly, Vero2-2 cells were co-transfected with pHSV$_S$[P12A3C] DNA, the BAC fHSVΔpacΔICP27 and the plasmid pEBHICP27, using Lipofectamine and Plus Reagent (Invitrogen, CA, USA). After 72 h, transfected cells were scraped into the medium, sonicated, and the cell debris was removed by centrifugation.

For titration, Vero 2-2 cells were infected with the amplicon vectors, and 24 h later, green fluorescent cells were counted using a fluorescence microscope (Axiovert S100, Zeiss). The titers were expressed as transducing units per milliliter (TU/ml).

For inactivation, 700 μl (5×10^6 TU/ml) aliquots of HSV$_S$[P12A3C] amplicon viral particles were treated with BEI (binary ethyleneimine) at a final concentration of 1.6 mM, during 12 h at RT. BEI was prepared as described [22].

2.5. FMDV protein synthesis in cells infected with HSV-derived amplicon vectors

Vero2-2 cells were infected with amplicon particles at a multiplicity of infection (MOI) of 1 TU/cell. After 6, 12 or 24 hpi, the cells were washed with PBS, lysed with 5× Laemmli buffer and separated on 15% SDS-PAGE. The fractionated proteins were transferred to nitrocellulose membranes, probed with the corresponding primary antibodies, and stained using anti-cow or anti-rabbit IgG antibodies conjugated with horseradish peroxidase, followed by Supersignal® West Pico Chemiluminescent Substrate (Pierce, Rock-

Table 1
Mice immunization schedule.

Group	Immunogen		Adjuvant	Dose (TU)	Inoculation route	Challenge ($10^{4.5}$ PFU)
	Prime (Day 0)	Boost (Day 28)				
1	HSV_S[P12A3C]	HSV_S[P12A3C]	–	5×10^5	ip	Yes
2	HSV_S[P12A3C]	HSV_S[P12A3C]	–	5×10^5	sc	–
3	HSV_S[P12A3C]	HSV_S[P12A3C]	–	5×10^5	im	–
4	HSV_S[P12A3C] inactivated	HSV_S[P12A3C] inactivated	–	5×10^5	ip	–
5	HSV_S[P12A3C] inactivated	HSV_S[P12A3C] inactivated	IFA	5×10^5	ip	–
6	HSV_S[VP6rv] inactivated	HSV_S[VP6rv] inactivated	–	5×10^5	ip	–
7	HSV_S	HSV_S	–	5×10^5	ip	Yes
8	O1/Campos	O1/Campos	FA/IFA	1 μg	ip	Yes
9	Ad[P12A3C]	Ad[P12A3C]	–	5×10^7	ip	Yes
10	HSV_S[P12A3C]	Ad[P12A3C]	–	$5 \times 10^5/5 \times 10^7$	ip	Yes
11	PBS	PBS	–	–	ip	–

FA: complete Freund's adjuvant; IFA: incomplete Freund's adjuvant. TU: transducing units; ip: intraperitoneal; sc: subcutaneous; im: intramuscular; rv, rotavirus; Ad: adenovirus; PFU: plaque forming units.

ford, USA), according to the manufacturer's instructions. Rabbit anti-VP1, anti-VP3 and anti-GFP polyclonal antibodies were used.

2.6. Immunofluorescence (IF) assays

Vero 2-2 cells were either mock infected or infected with the amplicon HSV_S[P12A3C] or HSV_S at a MOI of 0.03 TU/cell. At 24 hpi, the cells were washed once with PBS and fixed with 3.7% formaldehyde in PBS for 15 min at RT, and treated with 0.1 M Glycine in PBS for 5 min. Cells were permeabilized with 0.2% Triton X-100 for 15 min, washed with PBS, and blocked with 3% bovine serum albumin for 15 min. Cells were incubated for 1 h at RT with rabbit anti-VP1 or anti-VP3 antibodies at 1:200 dilutions. As secondary antibody, goat anti-rabbit IgG(H+L)-Alexa Fluor 594 (Invitrogen, USA) was used at 1:400 dilution. For visualization of the nuclei, the cells were incubated with 1 μg/ml DAPI (Roche, Switzerland) in PBS for 15 min at RT. Finally, the cells were washed three times with PBS and once with water and mounted on Glycergel (Dako Cytomation, Danmark) containing 25 mg/ml DABCO (Fluka, USA) to retard discoloration.

2.7. Immunoprecipitation with monoclonal antibodies

Extracts from HSV_S[P12A3C] infected Vero2-2 cells were incubated with either mouse preimmune polyclonal serum, or anti serotype O1 FMDV MAbs G8, 8G, 74 or 69 [23] for 2 h at 4 °C with gentle mixing. Fifty microliters of 50% protein A-Sepharose slurry were added and further incubated for 2 h, centrifuged at $1000 \times g$ for 1 min, and the resin was washed four times with Tris-buffered saline containing 0.1% Tween. The proteins were eluted in 2× Laemmli buffer and analyzed by SDS-PAGE and Western blotting. MAb G8 has neutralizing activity and was generated from O1/Campos strain, while MAbs 8G, 69 and 74, generated from O1/Caseros/Argentina/68 strain were shown to react with O1/Campos strain [23].

2.8. Purification of FMDV subunits

Amplicon vector infected HEK293 cells were lysed in NTE isotonic buffer (NaCl 100 mM, Tris–HCl 10 mM, EDTA 1 mM, pH 7.4) containing 0.1% (v/v) Triton X-100 (Sigma). After 10 min incubation, nuclei were removed by centrifugation and the supernatants were loaded onto a 5–30% sucrose gradient in NTE and centrifuged at 26,500 rpm for 6 h in a SW28 rotor at 4 °C. Density gradient fractions were collected and tested by solid phase ELISA (spELISA) using non-cross reactive anti FMDV serotype O1 MAbs, as previously described [23]. As a control, a stock of A24/Cruzeiro FMD virus strain was also run at the same conditions, and the 140S, 75S and 12S fractions were collected and reacted in spELISA, following the same protocol but using anti A24/Cruzeiro polyclonal rabbit antiserum and specific MAbs [23].

For electron microscopy, the fractions containing FMDV subunits were pelleted by centrifugation and adsorbed to carbon coated parlodion films and mounted on 300 mesh/in. copper grids (EMS, Fort Washington, PA, USA) for 10 min, washed once with H_2O, and stained for 1 min with 2% phosphotungstic acid pH7.0 (Sigma–Aldrich, Steinhein, Germany). Specimens were analyzed in a transmission electron microscope (CM12; Philips, Eindhoven, The Netherlands) equipped with a CCD camera (Ultrascan 1000, Gatan, Pleasanton, CA, USA) at an acceleration voltage of 100 kV.

2.9. Histopathological examination

BALB/c mice were injected with 5×10^5 HSV_S[P12A3C] or HSV_S amplicon particles or saline solution, via the im route. The animals were sacrificed 5 or 48 h later. The muscular tissue at the injection area and the popliteal lymph node were fixed in 4% formalin, paraffin-embedded, and stained with hematoxylin and eosin (HE) for histopathological examination, or mounted on positively charged slides for further processing. For immunohistochemistry, the slides were deparaffinated, counterstained with Hemalaun solution for 2 min, incubated with 3% H_2O_2/0.2% NaN_3 for 10 min at RT, and blocked with Protein Block Serum-Free (DAKO). Afterwards, the slides were incubated overnight at RT with rabbit anti-VP1 or VP3 antibodies at a dilution 1:200. As secondary antibody, an anti-rabbit serum conjugated with horseradish peroxidase (DAKO) was used, and amino-ethyl-carbazole (AEC, Invitrogen) was applied as chromogen. Between steps, the slides were thoroughly rinsed with PBS.

2.10. Analysis of immune responses in mice

The animal experiments have complied with all relevant federal guidelines and institutional policies. Groups of 4–8 weeks old BALB/c mice were immunized according to the schedule described in Table 1. Mice were boosted at 28 days post vaccination (dpv). Blood was collected from the submaxillar vein 1 week after each injection, and at 15-day intervals. The sera were clarified and stored at −20 °C.

Sera were tested for anti-FMDV antibodies by ELISA as described previously [23]. Immunoglobulin isotypes were analyzed by ELISA using suspensions of O1/Campos virus strain as capture antigen. Serial dilutions of serum derived from vaccinated mice were reacted with the antigen, followed by biotinilated anti-mouse IgG1 or anti-mouse IgG2a antibodies. The plates were subsequently incubated with an avidin-conjugated anti-mouse IgG(H+L) and developed with a substrate/chromophore mixture.

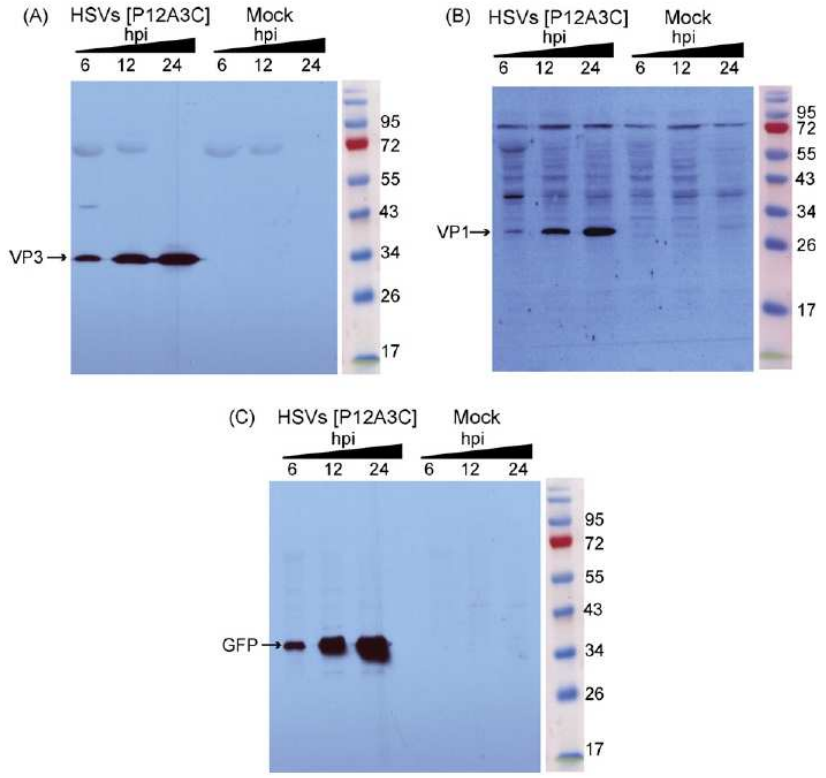

Fig. 2. Time course expression and processing of FMDV structural proteins. Protein expression and processing at 6, 12 or 24 hpi were analyzed by SDS-PAGE and Western blot using rabbit anti-VP3 (A), anti-VP1 (B), or anti GFP (C)polyclonal antibodies. The processed VP1 and VP3 proteins as well as GFP are indicated by arrows.

For lymphocyte proliferation assays, mice were sacrificed 3 weeks after the second immunization, and splenocytes were harvested. Briefly, 5×10^5 splenocytes were cultured in 100 μl of RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 50 mM β-mercaptoethanol, in 96-well U-bottom tissue culture plates (in triplicates). Cell suspensions were incubated with 100 μl of inactivated FMDV strain O1/Campos (10 μg/ml) or Con A (2.5 μg/ml). After 20 h, cultures were pulsed with 1 μCi/well of [^{3}H]thymidine, harvested 16 h later, and the [^{3}H]thymidine incorporation was counted in a liquid scintillation counter (Wallac1414 Win Spectral LSC). The stimulation index (SI) was calculated as the ratio of the average cpm value of wells containing antigen-stimulated cells to the average cpm value of wells containing non stimulated cells with medium.

2.11. Viral challenge

Groups of mice vaccinated with several different formulations, were challenged in BSL3A facilities with live O1/Campos virus, as described [24–26]. Briefly, vaccinated mice were intraperitoneally (ip) inoculated with $10^{4.5}$ $TCID_{50}$ of O1/Campos FMDV strain. After 24 h, animals were anesthetized and bled. Heparinized blood was serially diluted in culture medium and inoculated onto quadruplicate wells of BHK-21 cell monolayers, grown in 96-well plates. Cell monolayers were washed three times with sterile phosphate-buffered saline (PBS). Fresh DMEM containing 2% fetal calf serum (FCS) was added and the cells were kept for further 48 h at 37 °C in a 5%CO_2 incubator. The animals were considered fully protected when the cell monolayers did not present cytopathic effect (CPE) after a second blind passage (no viremia detected). Sera with positive viremia were titrated for quantification.

2.12. Statistical analysis

Statistical analyses were performed using SPSS software version 12.0 (Chicago, IL, USA). Comparison of humoral and cellular immune responses between groups was performed using an *a priori* nonparametric test (Kruskal–Wallis) followed by Mann–Whitney post test. *P*-values were considered to be significant if less than 0.05.

3. Results

3.1. Synthesis of FMDV proteins in mammalian cells infected with HSV_S[P12A3C]

An amplicon plasmid that enables simultaneous expression of FMDV proteins and GFP in the infected cells was constructed (Fig. 1). The EGFP gene was introduced to allow the titration of the vector stocks by fluorescence. Amplicon vector stocks, prepared using a helper virus free packaging system [21], had titers of around $1–5 \times 10^6$ TU/ml.

FMDV capsid polyprotein was synthesized in Vero 2-2 cells infected with HSV_S[P12A3C], and processed into the expected products. Time course synthesis of the different proteins was studied at 6, 12 and 24 hpi. Processed VP3 and VP1 proteins were detected in Western blots at 6 hpi, and their levels increased with time (Fig. 2, panels A and B, respectively). The same expression pattern was found for GFP (Fig. 2, panel C). Increased CPE was observed in infected cells (data not shown), possibly due to the toxic effect of 3C protease. Similar results have been reported in other expression systems [27].

The expression and location of the recombinant proteins in Vero2-2 cells infected with HSV_S or HSV_S[P12A3C] amplicon vectors were also examined by indirect IF using a confocal microscope.

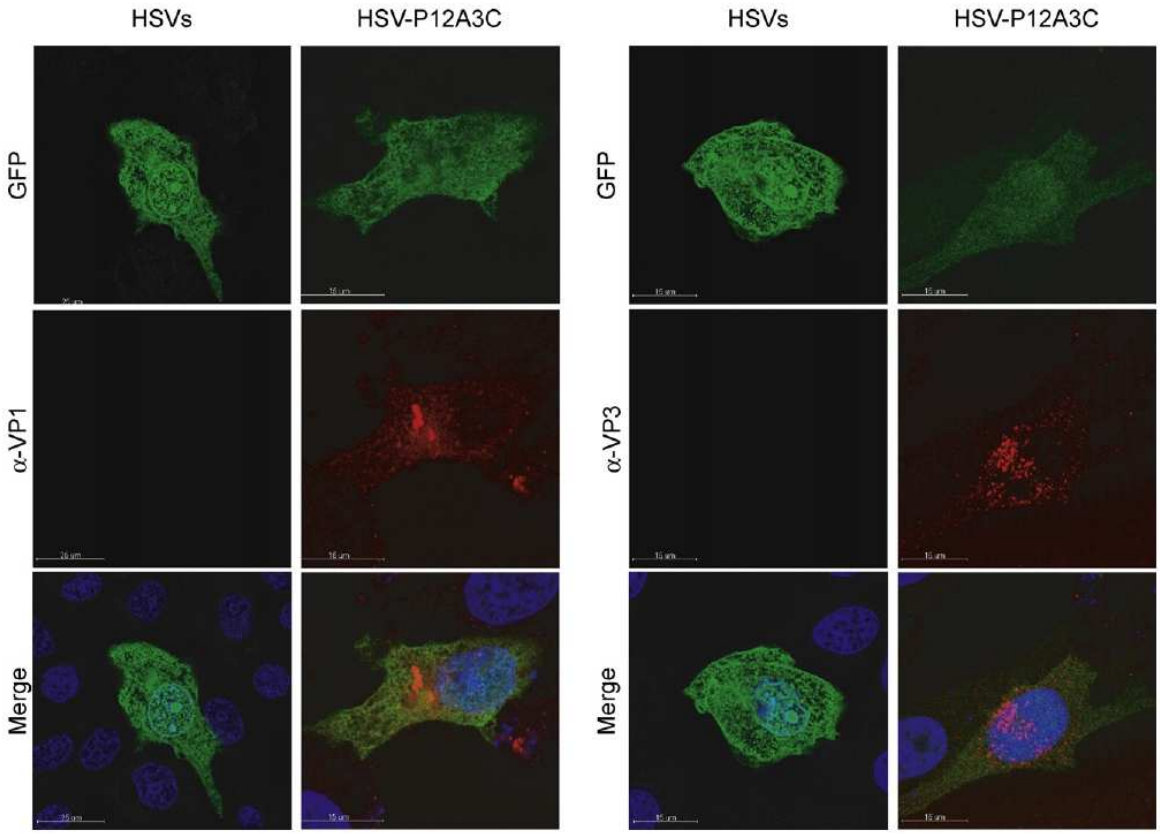

Fig. 3. Intracellular expression of FMDV VP1 and VP3, and GFP expressed by HSV-1 derived amplicons. Vero2-2 cells infected with HSV_S[P12A3C] or HSV_S amplicons, as indicated at the top of the figures, were fixed, permeabilized and probed with anti-VP1 or anti-VP3 rabbit antisera, as indicated to the left of the figures. GFP fluorescence was observed in cells transduced with both amplicons. DNA was stained with DAPI. The merge of the images is shown in the bottom panel.

Specific GFP fluorescence was observed in cells infected with both amplicons (Fig. 3). In cells infected with the HSV_S[P12A3C] amplicon, IF staining of VP1 or VP3 showed an homogeneous red pattern within the cytoplasm. No specific fluorescence for VP1 or VP3 proteins was observed in cells infected with HSV_S and treated with anti-VP1 or anti-VP3 serum.

Cells infected with HSV_S[P12A3C] exhibited a reduced EGFP fluorescence compared to HSV_S infected cells (Fig. 3), which might be due to the increased distance between the EGFP cistron placed after an IRES structure and the IE 4/5 promoter in the former vector.

The FMDV subunits produced in HSV_S[P12A3C] infected cells were further characterized using specific FMDV MAbs. Lysates of cells infected with HSV[P12A3C] were immunoprecipitated with the MAb G8, which recognizes a conformation-dependent neutralizing epitope on VP1 located on the surface of intact FMDV O1/Campos particles. MAb G8 precipitated VP1 as well as VP0 and VP3, as revealed by subsequent Western blot analysis of the immunoprecipitated products (Fig. 4). Similar results were observed with MAbs 8G, 74 and 69. These results indicated that the MAbs recognized aggregates containing the three proteins, with structural antigenic sites similar to intact capsids.

3.2. Characterization of capsid-like particles assembled in HSV_S[P12A3C] infected cells

Sedimentation in 5–30% sucrose gradients was used to analyze the ability of FMDV capsid proteins encoded from HSV-1 amplicon vectors to assemble into particulated structures or subunits. A small fraction of the purified proteins sedimented at the same rate (75S) as native empty FMDV strain A24/Cruzeiro capsids, which were used as standard (Fig. 5A). It is worth mentioning that O1/Campos was not used as a standard because it does not produce native empty particles in our culture system [23]. A well defined peak was identified close to the top of the gradient, with a sedimentation coefficient similar to the 12S native viral subunits. Interestingly, an unusual component sedimenting at a lower rate than empty capsids (20S) was also detected. This sedimentation pattern did not vary between experiments, as it was always possible to detect the three described components. Furthermore, [^{35}S]-methionine protein labeling and immunoprecipitation experiments revealed that VP0, VP1 and VP3 were directly interacting in the 75S, 20S and 12S subunits (data not shown).

Analysis of the fractions by electron microscopy revealed the presence of naturally occurring empty VLPs of approximately 30 nm in diameter (Fig. 5B), which were similar to natural viral empty particles. Pentameric capsomers (approximately 12 nm) and large amounts of capsomer aggregates were also detected.

To estimate the number of FMDV VLPs assembled in HSV_S[P12A3C] infected cells, the empty particles adsorbed on the grids were counted. The production was roughly estimated at 5 VLPs per infected cell in an 8 h infection cycle. The copy number of $pHSV_S$[P12A3C] DNA in each amplicon was estimated at 15.

3.3. In vivo protein expression

The expression of FMDV transgenes in BALB/c mice injected with 5×10^5 TU of HSV_S[P12A3C] was investigated. At different time points after HSV_S[P12A3C], HSV_S or HBSS injection, mice were sac-

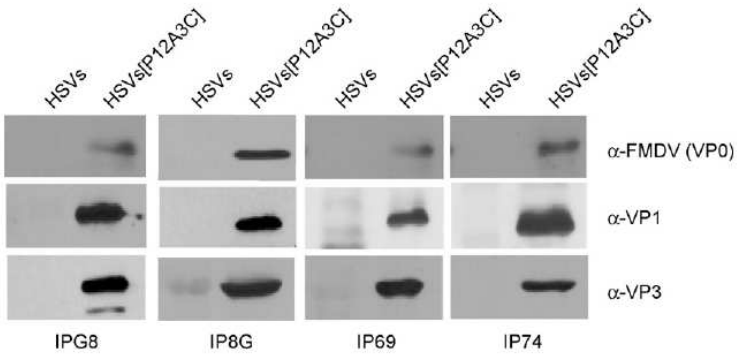

Fig. 4. Immunoprecipitation assays. Total cells extracts from Vero 2-2 cells infected with either HSV_S[P12A3C] or HSV_S amplicons, were immunoprecipitated with FMDV serotype O1 specific MAbs G8, 8G, 69 and 74, separated by SDS-PAGE and further analyzed by Western blot with rabbit anti-VP1, anti-VP3 or anti-FMDV polyclonal sera, as indicated. IP: immunoprecipitation.

rificed and the muscle and popliteal lymph nodes were removed and analyzed for inflammatory reactions, as well as FMDV antigen. At 5 hpi, a slight neutrophilic and histiocytic infiltration was visible at the injection site of all mice (data not shown). At 48 hpi, a severe focal-extensive histiocytic and neutrophilic infiltration was observed in mice injected with HSV_S[P12A3C] amplicons, while mice injected with HBSS or HSV_S showed a slight to moderate inflammatory process. In most animals, a few muscle fibers showed

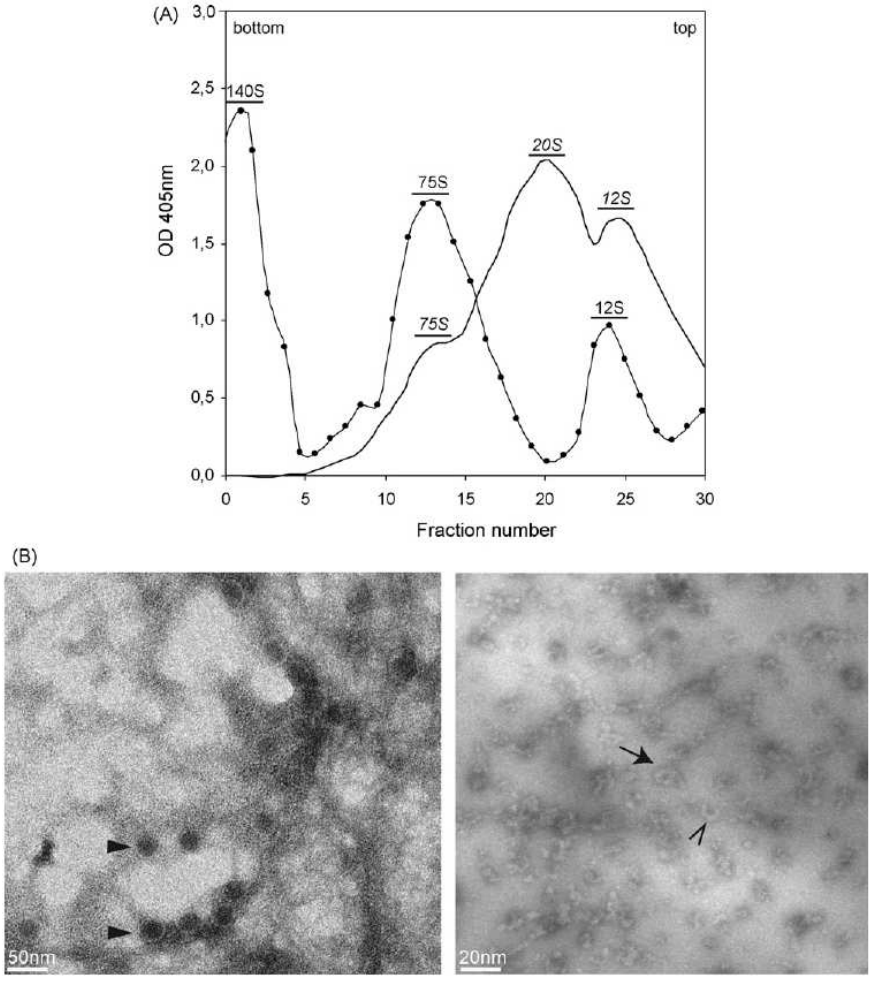

Fig. 5. Analysis of FMDV subunits. (A) Sucrose density gradient (5–30%) of extracts of cells infected with HSV_S[P12A3C]. The fractions were analyzed by ELISA using specific monoclonal antibodies and the absorbance values were measured at 405 nm. The density gradient regions corresponding to the known positions of the intact FMDV strain A24 Cruzeiro particles (140S), empty particles (75S) and capsomers (12S) are indicated (dotted line). The positions of the recombinant FMDV subunits are also indicated (full line). (B) Electron micrograph of the recombinant FMDV subunits fractions showing empty capsid particles (left) and capsomers and polymorphic particulated aggregates (right). Scale bar = 50 nm or 20 nm. ◄, Virus-like particles; >, capsomer; →, capsomer aggregates.

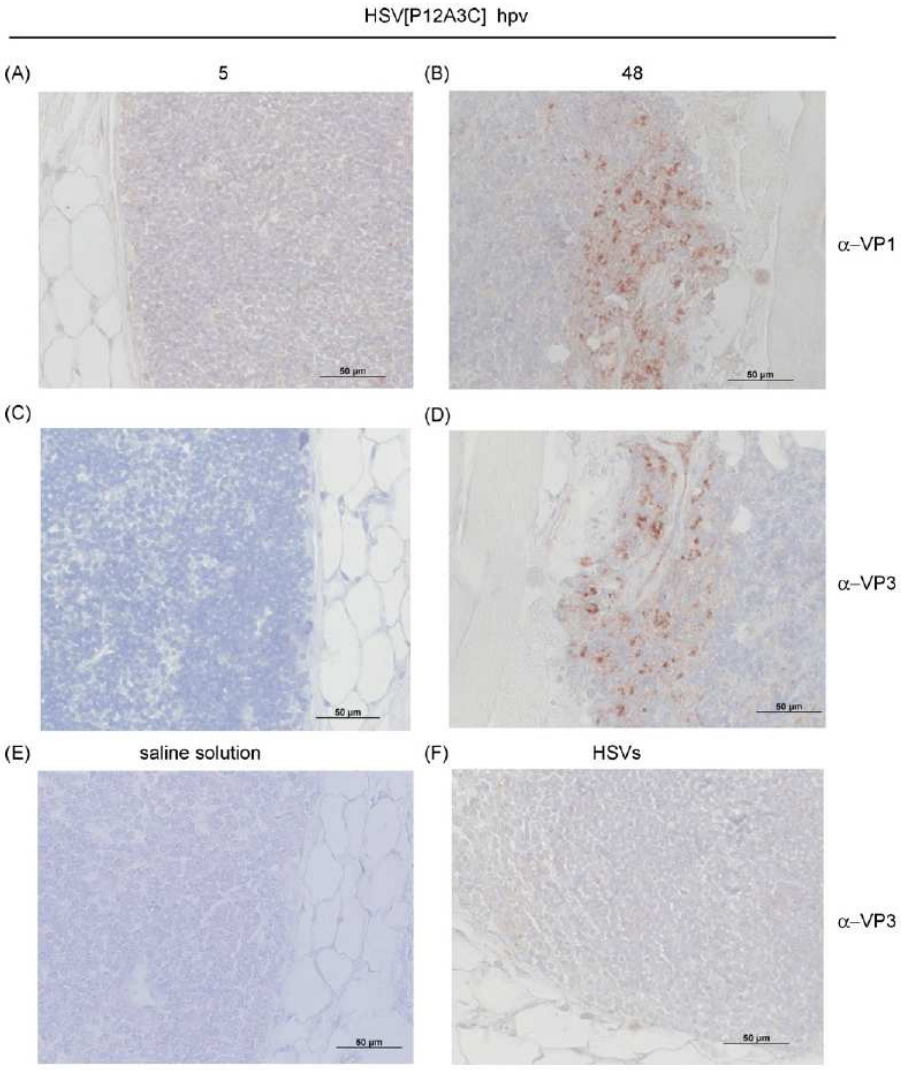

Fig. 6. Detection of FMDV proteins in tissue of mice inoculated with HSV-1 amplicons. Cryosections taken from the popliteal lymph nodes of BALB/c mice, removed at 5 (A and C) or 48 (B and D) h post intramuscular injection with 5×10^5 TU of HSV_S[P12A3C], were immunostained with anti-VP1 (A and B) or anti-VP3 (C and D) antibodies. Cryosections from mice injected with HBSS (Hanks' Balanced Salt Solution) or HSV_S, removed and stained at 48 hpi with anti-VP3 antibodies are shown in panels E and F. hpv: hours post vaccination.

degeneration and macrophage infiltration, probably as a consequence of the injection itself. The popliteal lymph nodes of all the animals examined showed normal structure, without secondary follicles.

Cells positively stained with VP1 and VP3 antibodies were visible at 48 hpi in the popliteal lymph nodes of mice injected with HSV_S[P12A3C] (Fig. 6B and D, respectively), while they were negative at 5 hpi (Fig. 6A and C). Positive cells were located in the area of the marginal sinus. The lymph nodes of mice injected with HBSS or HSVs remained negative (shown for VP3 antiserum in Fig. 6, panels E and F).

3.4. Immune responses generated with HSV_S[P12A3C] amplicon vectors in mice

Sera from BALB/c mice injected and boosted with either 5×10^5 TU HSV_S[P12A3C] or HSVs amplicons, 5×10^7 TU Ad[P12A3C], PBS or inactivated FMDV strain O1/Campos in complete Freund's adjuvant, according to the scheme shown in Table 1, were collected and analyzed.

Anti-FMDV end point ELISA was performed with serum from individual animals bled at 7, 21, 35 and 50 days post vaccination (dpv). Mice inoculated with HSV_S[P12A3C] induced FMDV-specific antibodies at 21 dpv, which increased greatly after the booster immunization. Statistical analysis indicated that the antibody titers of ip vaccinated mice were significantly higher ($P < 0.05$), compared to the groups of mice immunized by the intramuscular (im) or subcutaneous (sc) routes (Fig. 7A). Interestingly, similar antibody titers were observed in mice immunized ip with non-adjuvanted HSV_S[P12A3C] or with inactivated oil-adjuvanted O1/Campos virus. Mice inoculated with 10^7 TU of Ad[P12A3C] showed lower serum titers levels (data not shown).

The presence of small amounts of preformed antigen in HSV amplicon preparations may contribute to the immune response

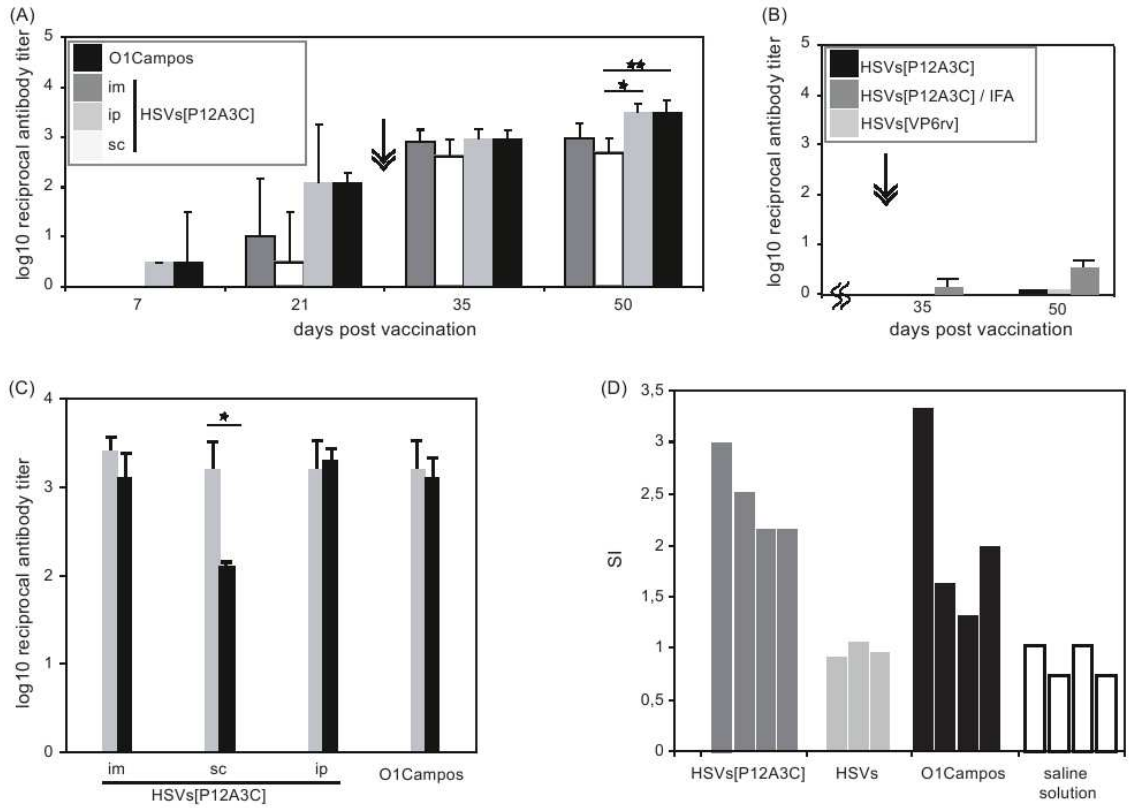

Fig. 7. Immune responses in mice after administration of HSV_S[P12A3C] amplicon particles. (A) Kinetics of antigen-specific antibodies in BALB/c mice. The arrow indicates the booster inoculation. The asterisks indicate statistical significance in antibody titers ($P < 0.05$). (B) Kinetics of antibodies in BALB/c mice inoculated ip with 5×10^5 TU of BEI-inactivated HSV_S[P12A3C] amplicon particles, with or without incomplete Freund's adjuvant (IFA). HSV_S[VP6rv]: inactivated amplicon expressing a non related antigen (rotavirus protein VP6). FMDV-specific antibody titers were determined by ELISA in sera from animals bled at 35 and 50 dpv. (C) Isotype antibody profile induced by different immunization routes. ELISA titers of anti-FMDV specific IgG1 (gray) or IgG2a (black) determined at 50 dpv are shown. The asterisk indicates the statistically significant difference between IgG1 and IgG2a levels in mice vaccinated sc ($P < 0.05$). (D) Lymphocyte proliferative response, measured at 21 days of booster inoculation, after stimulation with inactivated FMDV O1/Campos (10 μg/ml). Incorporation [^{3}H]thymidine was determined and the mean value of the cpm on triplicate wells was used to determine the stimulation index (SI). Data represent the mean SI obtained for individual mouse in each group.

found in vivo [28]. In order to analyze the contribution of preformed protein to the immune response found in vivo, BALB/c mice were inoculated and boosted with the same amount of BEI inactivated HSV_S[P12A3C] amplicons, either in oil adjuvant or alone (Table 1). FMDV specific antibody titers were analyzed at 35 and 50 dpv (Fig. 7B). Inactivated amplicons in the absence of adjuvant did not elicit an immune response to FMDV proteins, while in the presence of adjuvant they induced an antibody response, but with titers substantially lower than those produced by non-inactivated HSV_S[P12A3C] amplicons (Fig. 7A and B). These results suggest that preformed FMDV structural proteins do not significantly contribute to the specific FMDV antibody response induced in mice by the amplicons. The same amount of an inactivated amplicon, HSV_S[VP6rv], expressing a non related antigen (rotavirus protein VP6) was also included in the experiment in order to show that the immune response is specific, and not mediated by the carrier itself when another antigen is expressed.

Analysis of the Ig isotypes revealed that immune sera from mice immunized ip or im with HSV_S[P12A3C] generated a mixed IgG1/IgG2a immune responses, while immunization via the sc route induced IgG1 as the predominant isotype ($P < 0.05$, Fig. 7C).

To investigate whether inoculation with the HSV_S[P12A3C] amplicons could also enhance cell-mediated immune responses, mice were sacrificed 3 weeks after the second immunization. Splenocytes were harvested and lymphocyte proliferative assays were performed using O1/Campos inactivated virus for stimulation and Con A as positive control. As indicated by the stimulation index, the proliferative response was significantly higher in mice immunized im with HSV_S[P12A3C] than in mice immunized with HSVs ($P < 0.05$), and similar to that obtained in mice inoculated with inactivated virus (Fig. 7D).

3.5. Challenge of mice with live O1/Campos FMDV strain

Mice in groups 1, 7, 8, 9 and 10 (Table 1) were challenged in BSL3A facilities with a high dose ($10^{4.5}$ $TCID_{50}$) of live O1/Campos virus strain and analyzed for the presence of viremia 24 h post challenge (hpc). Two other groups of mice vaccinated twice with Ad[P12A3C] (group 9) or primed with HSV_S[P12A3C] and boosted with Ad[P12A3C] (group 10), were identically challenged.

Sera from these mice taken before challenge were analyzed by ELISA. The highest antibody titer level corresponded to group 1 vaccinated with HSV_S[P12A3C], followed by mice inoculated with inactivated virus (group 8), and by mice boosted with Ad[P12A3C] (group 10) (Fig. 8A). The highest reduction of viremia was found in the inactivated virus vaccinated mice, followed by the HSV_S[P12A3C] group (Fig. 8B), as compared to the empty amplicon ($P < 0.05$).

It is worth mentioning that total protection (as estimated by the lack of viremia after two subsequent blind passages) was found in

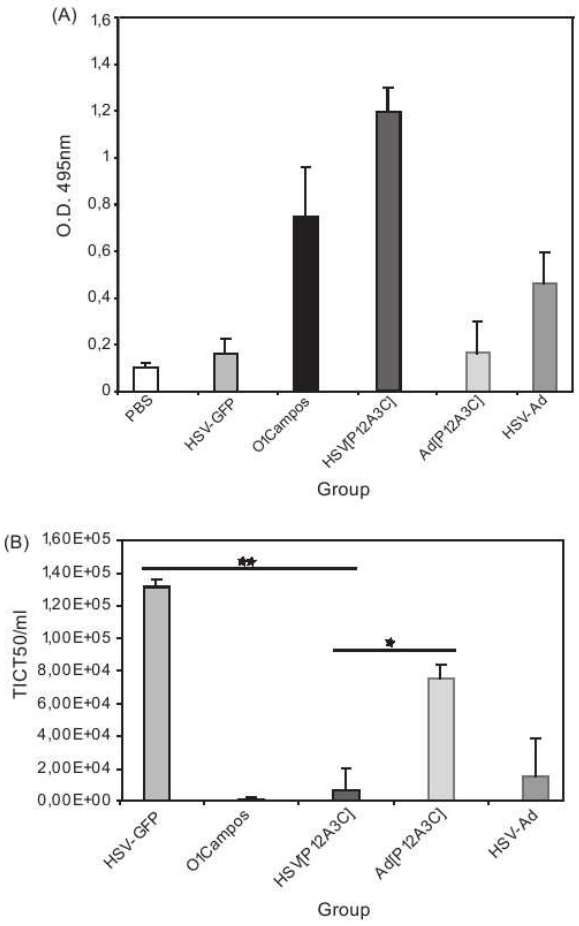

Fig. 8. Challenge experiments in mice. BALB/c mice belonging to the groups 1, 7, 8, 9, and 10 described in Table 1, were vaccinated twice, as described, and challenged with $10^{4.5}$ $TCID_{50}$ of infectious O1/Campos FMDV strain. (A) Serum samples taken before challenge were analyzed by ELISA. (B) Serum samples taken 24 h post challenge were analyzed for circulating virus (viremia) by titration on BHK-21 cells in quadruplicates. The asterisks indicate statistical significance in the reduction of viremia ($P < 0.05$), in the indicated groups.

two mice (2 out of 6) of the HSV_S[P12A3C] group and in one (1 out of 5) mouse of the inactivated virus group (data not shown). The low number of animals totally protected from viremia at 24 hpc was probably due to the high viral challenge dose used in the experiment ($10^{4.5}$ $TCID_{50}$ per mouse).

Mice with homologous HSV_S[P12A3C] prime-and-boost (group 1) elicited higher antibody titers and protection than the homologous Ad[P12A3C] primed and boosted (group 9, $P < 0.05$), although the number of particles of Ad[P12A3C] inoculated was 100-fold higher. The animals primed with HSV_S[P12A3C] and boosted with Ad[P12A3C] (group 10) gave an intermediate result (Fig. 8B).

4. Discussion

In the present study, bicistronic HSV-1 amplicon vectors were used to co-express FMDV structural proteins and the reporter EGFP gene, via the use of an IRES structure. The heterologous proteins were detected in amplicon-transduced cells as early as 6 hpi, presumably reflecting the strong promoter used and the large transgene capacity of the HSV-1 amplicons, which in this case afforded the encapsidation of approximately 15 copies of the transgenes.

The assembly of FMD VLPs, as well as other smaller particulated structures sedimenting as 20S and 12S was observed. Preliminary quantification studies estimated that, in the conditions used, around 5 VLPs per infected cell were produced. Co-immunoprecipitation analysis and reactivity with neutralizing MAbs that recognize intact 140S FMD virions suggested that the VLPs retained natural antigenicity.

The helper virus-free HSV-1 amplicon vector system described in this work, which combines the high immunogenicity of modified live virus vaccines with the safety and simplicity of DNA vaccines, was able to induce both humoral and cellular immune responses in preliminary experiments. Interestingly, mice vaccinated twice with 10^5 TU of HSV_S[P12A3C] amplicon particles elicited a similar or higher overall IgG response than mice vaccinated twice with 1 μg of inactivated purified virus in Freund's adjuvant. This is highly promising, since it has recently been reported that a boosted inoculation of 1.2 μg of FMDV type Asia 1 VLPs produced in baculovirus induced significantly lower antibody levels in guinea pigs than similar amounts of intact natural viral particles produced in BHK cells [5].

Although only limited challenge experiments were performed, it was highly promising that using a high challenge dose of $10^{4.5}$ $TCID_{50}$ of infectious virus (similar to the bovine challenge dose), mice vaccinated with HSV-derived amplicons showed a significant reduction of viremia (Fig. 8B), close to the mice vaccinated with conventional vaccine, and moreover, two of the mice were totally protected from viremia at 24 hpc. In our hands, the performance of the HSV-derived vectors was better than the Ad-derived vectors (inoculated dose: 10^5 and 10^7 TU, respectively). This might be due to the presence of multiple copies of the capsid proteins coding region encapsidated by the HSV amplicons. Preliminary data from the homologous or heterologous boosts carried out with these vectors (groups 1, 9 and 10) did not reflect a negative influence of preexisting immunity against the HSV-1 vector itself in the mice model used. In this sense, double vaccination with HSV-1 derived vectors worked better than vaccination with herpetic amplicons boosted with by a heterologous (Ad) vector.

HSV_S[P12A3C] amplicon particles elicited antibodies of the IgG1 and IgG2a subclasses, suggesting the generation of a mixed Th1- and Th2-type response in mice. This could conceivably be due, at least in part, to the ability of these vectors to produce soluble extracellular FMDV proteins in vivo, in addition to intracellular antigens. On the other hand, the delivery system itself may influence the nature of the immune response that is elicited. In fact, infection of both ICR and BALB/c strains of mice with wild-type HSV-1 results in a humoral immune response that is predominantly of the IgG1 subtype, but with substantial levels of IgG2a [29,30]. Thus, HSV-1 itself appears to elicit a mixed Th1/Th2 response, as do the amplicon vectors. Preliminary results also indicated that a lymphoproliferative response was induced in mice splenocytes (Fig. 7D).

The humoral immune response generated in mice was mainly elicited by antigen generated by *de novo* synthesis in infected cells as BEI inactivated amplicons elicited a very low measurable immune response only when inoculated with oil adjuvant. In agreement with these results, work performed with HSV-1 amplicons expressing the HIV-1 Gag protein, reported that preformed antigen decays rapidly in vivo and accounts only for a minimal contribution to the total antigenic mass inoculated [28].

HSV-1 amplicon vectors have several advantages over other vector systems. They are devoid of HSV-1 genes, infect many different cells including dendritic cells and other potential antigen-presenting cells, have very low toxicity [20,21], and they possess a very large transgene capacity (up to 150 kb), which allows the incorporation of multiple transgenes or multiple copies of a transgene [1]. The ability to generate multicistronic vectors with additional insertion of IRES structures (Laimbacher A,

D'Antuono et al., unpublished) or additional promoters, holds an interesting potential for the development of polyvalent FMDV vaccines expressing capsid proteins of two or more different FMDV serotypes, especially taking into account the large capacity of these vectors. Moreover, HSV-1 amplicons can accommodate multiple transcription units to co-express selected antigens and different cytokines or immunomodulatory molecules.

The production of helper virus-free amplicons using DNA transfection procedures was rather low, and the vector stocks cannot be further amplified. At present, no method combining a total absence of contaminating helper particles with ability to produce very large amounts of amplicon particles is available. However, other packaging systems have been developed which allowed the production of high titer safe amplicon stocks that contain 0.05–1.0% of contaminating defective helper viruses [31,32], which may not constitute a major problem in animal vaccines.

It is worth mentioning that from the prospective of FMD alternative vaccines, the significance of the HSV-1 amplicon system is highly dependent on the results of protection studies performed in the target species. This has not been addressed at this time, because of the regulatory and economical complexity of those experiments. We are currently exploring the possibility of amplicon administration, alone or combined with conventional vaccination, so as to provide long lasting protection as well as immunity at the primary portal for virus entry (i.e. the oro-pharynx).

The preliminary results obtained with HSV-1 amplicons for the in situ production of FMDV VLPs in mammalian cells are encouraging for the development of vaccine prototypes employing this platform system for this virus or for other pathogens.

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3 Perspectives

The aim of this study was to evaluate the feasibility of HSV-1 based amplicon vectors as genetic vaccine candidates. Two RNA viruses, rotavirus and Foot-and-mouth disease virus (FMDV), both of which cause severe diseases, have been chosen for this work. At present, most of the currently licensed vaccines are based on live attenuated or inactivated viruses or subunits of the virus particle. Besides safety concerns and the need of high-risk facilities for the production of the vaccine strains, which makes the vaccines expensive, the viruses studied in this thesis consist of a RNA genome. RNA viruses are known to mutate fast in a relatively short time. This high antigenic variation and the already existing amount of circulating serotypes of the two viruses makes it even more important to generate a vaccine platform, which makes it possible to create in a relatively short time efficient and safe vaccines.

In the present work, HSV-1 amplicon vectors have been designed to support the in situ production of virus-like particles (VLPs) in the vector-transduced cells. VLPs are very promising vaccine candidates, as they are structurally similar to the infectious virus, but they lack viral nucleic acid, are non-infectious and are therefore totally safe. Thus, they combine many of the advantages like the high antigenicity of the viral capsid (inactivated or attenuated whole-virus vaccines) with a high safety level (viral subunit vaccines). Besides these convincing advantages, there are major limiting factors concerning the use of VLPs as vaccines candidates, including the high production and purification costs to obtain the large amounts of highly purified and stable particles needed for large clinical trials. The chosen strategy to produce VLPs directly in the vector-infected cells or organism has the immense advantage that there is no need to purify the produced VLPs prior to injection. Moreover, when compared to injection of purified VLPs, delivering the genes into the cells results in the intracellularly production of the antigen, and the immune response generated mimics that induced by replication of live pathogen, which could lead to development of both humoral and cellular responses. This feature is also true for DNA vaccination, but using HSV-1 amplicon vectors for the delivery of the antigen needs no addition of an adjuvant (1). The baculovirus system is well established and known to generate high levels of foreign proteins, but the posttranslational processing of proteins in insect cells greatly differs from that in mammalian cells. Using mammalian cells for vaccine vector production supports the appropriate modifications and authentic assembly of the VLPs, resulting in even better mimicking of the wild-type virus strain.

On the other hand, a critical point of using HSV-1 amplicon vectors for the generation of vaccines against RNA virus infections is the fact that HSV-1 replicates in the nucleus, whereas both studied RNA viruses do not enter the nucleus of the infected cells. This

concern was addressed in the current work on rotavirus vectors and no impact on the protein level or number of VLPs produced could have been demonstrated, although aberrant splicing events have been identified.

HSV-1 amplicon vectors are easy to construct, and their large transgene capacity allows the incorporation of several genes of interest, up to about 150 kb, and the encapsidation of several copies of the transgene cassette. The helper virus-free system used in this study allows the production of vector stocks that are free of contaminating HSV-1, providing a high safety level of these vectors. The limiting factor when using this BAC-based system is the relatively low amount of resulting vector stocks. In the case of FMDV, toghether with our collaborators in Argentina, we immunized cows with the amplicon vectors (not described in this thesis). The amount of vector used for the immunization was $1x10^7$ TU per injection. The same prime and boost regimen was used as previously in mice. Upon challenge, none of the three vaccinated cows was protected and, none of the cows produced virus-specific antibodies. From this preliminary experiment we concluded that the amount of injected antigen was simply not enough to elicit a detectable antibody response, as researchers using replication-deficient adenoviral vectors used doses of $5x10^9$ pfu per animal (summarized in (5)). Therefore, the need of a more efficient packaging system, which still produces helper-free stocks, but which yields in higher amounts of vectors is of great importance for future possible application in larger trials and in other animals than mice.

The transgene cassettes used in this work were shown to efficiently express the viral genes of interest, leading to the in situ generation of VLPs in the vector-infected cells. Therefore it could be an alternative to use another virus platform, but the same strategy of generating VLPs in the vector-infected organism, specifically to use the transgene cassettes characterized in this study. Such a candidate virus could be the Orf virus (ORFV), which belongs to the Genus Parapoxvirus of the family *Poxviridae*. Orf virus based vectors, which express foreign antigens, have been shown to be immunogenic and provide protection against several viruses in a number of animal models. This includes a study performed in mice with pseudorabies virus (4), in rats with borna disease virus (7) and in pigs with classical swine fever virus (12) and pseudorabies virus (2).

FMD is a highly contagious viral vesicular disease of cloven-hoofed animals that generally causes severe economic losses. FMDV affects mostly cattle, pigs and sheep, but it can infect and cause disease to more than 70 domestic and wild animal species (6). The high economic impact of FMD is based on virological characteristics of the causing virus, like high speed of replication, short incubation time, high contagiousness because of high level of virus excretion via aerosols and a high mutation rate resulting in constant antigenic changes. If the virus is not controlled quickly and efficiently, it spreads extremely rapidly in a susceptible population (11).

Seven distinct FMDV serotypes have been described so far. Immune responses induced by FMDV infection or vaccination with one serotype do not cross-protect against other serotypes, and in many cases, protection may be restricted only to closely related strains within the same serotype. The current available vaccines are based on chemically inactivated whole virus preparations used in conjunction with an adjuvant (5), which are very successful in reducing the number of disease outbreaks and the spread of the virus during an outbreak, but there are some concerns and limitations of this inactivated FMDV vaccine: (i) The need of expensive high-containment facilities required for the production of the vaccine, (ii) The difficulties to distinguish between vaccinated and infected or convalescent animals with currently approved diagnostic tests, as the vaccine contains contaminating viral non-structural proteins, (iii) The vaccine does not induce rapid protection against challenge by direct or indirect contact resulting in a 7 to 14 days window of susceptibility of the vaccinated animals and (iv) vaccinated animals can become long-term carriers following contact with FMDV (5, 9). There are three different states with regard to FMD according to The World Animal Health Organisation (OIE, office international des epizooties): (i) FMD present with or without vaccination, (ii) FMD free with vaccination and (iii) FMD free without vaccination. Only countries stated FMD free without vaccination possess full access to the international trade. For a country to be considered free of FMD and therefore to regain full access to livestock trade, it is crucial to distinguish between antibodies generated during a natural infection and from those produced after vaccination (6). FMD is monitored based on serological tests and both, natural infection and vaccination with conventional inactivated vaccines results in a seropositive animal, which is the reason why these vaccines are not routinely used, as the country would loose its disease-free status. Also, the vaccinated animals have to be slaughtered to re-establish the FMDV-free status (3).

To overcome the above described concerns related to the existing vaccines, two major points need to be fullfilled for a new vaccine to be successful: providing rapid protection to limit virus spread and being able to distinguish vaccine-inoculated animals from infected animals. One widely studied approach is to use a replication-defective human adenovirus to deliver the parts of the genome required for empty capsid synthesis and assembly, the protein precursor P1-2A and the protease 3C (5, 8, 10), the same region also used for this study.

The development of the amplicon vectors decsribed in this work provides another opportunity to design recombinant FMDV vaccines that have the advantages of safety, authentic antigenicity and most important, differentiation of infected from vaccinated animals by the lack of non-structural proteins like the 3D polymerase compared to infected animals. By using VLPs, there is no antibody response to internal or non-structural viral proteins induced, which allows the serological distinction between vaccinated and infected animals using

suitable diagnostic tests. The results obtained in mice in the present work are very promising as upon challenge with a high dose of virus, mice vaccinated with the amplicon vectors showed a reduction of viremia close to the animals vaccinated with the conventional vaccine. Also, the protection was better when using the amplicon vectors than adenovirus vectors.

To summarize the presented work, the obtained results provide good evidence for the potential of HSV-1 amplicon vectors to be used for the development of a new vaccine strategy against FMDV and Rotavirus infections.

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5 Curriculum vitae

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Education & Employment	
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04 / 2006	Diploma in Zoology, University of Zurich
since 07 / 2006	PhD Student, Science Faculty, University of Zurich
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Languages	German (mother tongue) English
Research projects	
10 / 2004 – 01 / 2006	**Institute of Virology, Vetsuisse Faculty, UZH** Diploma student Supervisor: Prof. M. Ackermann
	Diploma project: Open reading frame O9 of ovine herpesvirus 2 encodes a Bcl-2 homolog which targets the mitochondria
since 07 / 2006	**Institute of Virology, Vetsuisse Faculty, UZH** PhD student Supervisor: Prof. C. Fraefel
	own research projects: HSV-1 based vectors encoding rotavirus and foot-and-mouth disease virus structural proteins Toolbox for serological detection of influenza virus infection
Teaching experiences	
	Co-Supervision of student courses for Biology Students from University of Zurich and ETH Zurich Supervision of Master Students, Academic Guests, Trainee for Technician Co-Supervision of Training Course in HSV- based vector techniques: for Master and Graduate Students from South America
Further education	
	Labortierkundekurs LTK Modul 1 Confocal Microscopy Course